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Antioxidant and antimicrobial activities of extracts prepared from fruit and vegetable wastes and by-products

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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF EXTRACTS PREPARED FROM FRUIT AND VEGETABLE WASTES AND BY-PRODUCTS

（農産廃棄物及び農産加工副産物由来抽出物の抗酸化性と抗菌性）

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN AGRICULTURAL SCIENCE IN THE INTERDISCIPLINARY GRADUATE SCHOOL OF SCIENCE AND TECHNOLOGY AT THE SHINSHU UNIVERSITY

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ABSTRACT

The functional food constituent of human diet mainly comes from fruits and vegetables and these are the major dietary source of nutraceuticals. The aim of the present study was to evaluate the functionality of extracts prepared from fruit and vegetable wastes and by-products. In the present study, under-utilized fruit and vegetable wastes and by-products extracts were tested for their antioxidant as well as antimicrobial properties. Hot-water and ethanol were used for extraction processes. Antioxidant activities were evaluated based on the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay and the AAPH [2,2'-azobis- (2-amidinopropane) dihydrochloride]-induced linoleic acid (LA) peroxidation test, whereas antimicrobial effects were determined using agar plate count and spectrophotometric assays. Most extracts prepared from fruit and vegetable wastes and by-products exhibited a potent antioxidant activity in DPPH free radical and AAPH peroxyl radical. The highest level of antioxidant activities were detected in grape seed, in addition immature prune and buckwheat hull exhibited efficient antioxidant activities with DPPH free radical. A positive correlation was observed between antioxidant activities and phenolic contents of extracts. Thus, fruit and vegetable wastes and by-products are the potential source of natural antioxidants. In antimicrobial assay, the extracts from fruit and vegetable wastes and by-products showed bacteriostatic as well as bactericidal effects, whereas Gram-positive bacteria were more susceptible than the -negative. A moderate growth inhibition was observed in immature prune and peach, whereas strong effects were detected in grape bunch stem followed by grape wine pomace, and Chinese quince sake pomace. The highest level of antimicrobial effect was exhibited by the grape bunch stem extract. The results showed that the bioactive components of agricultural wastes and by-products having antioxidant as well as antimicrobial potency. In addition, the mode of action of antimicrobial activities depends on the type of microorganisms and their cell wall structures. In contrast, it was demonstrated that CGA and related compounds exhibited a potent antimicrobial activities with the synergistic effects. Crude extracts from fruit wastes and by-products could be a potential source of antimicrobial candidacy. Thus, Fruit and vegetable wastes and by-products are the potential sources of natural bioactive compounds. The exploitation of these abundant and low-cost renewable resources could be anticipated for the food industries during packaging and/or storage. As a conclusion, it could be said that utilization of functional food components from these extracts will improve agricultural sustainability by maximizing the use of agricultural wastes and by-products.

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SUMMARY

Recently, the researcher have given importance on plant and vegetable extracts, as well as extracts prepared from agricultural wastes and by-products as alternative forms of nutraceuticals, which are ongoing demand all over the world to satisfy the increasing demand of emerging population of the world. Thus, the research of the thesis deals with the functional constituents of agricultural wastes and by-products.

In Chapter 1, background and perspective of research on agricultural wastes and by-products have been elicited. The objectives and thesis outline have also been stated.

In Chapter 2, total polyphenols content and antioxidant activities of crude extracts prepared from fruit and vegetable wastes and by-products were investigated. It was indicated that the crude extracts contain high amount of phenolics with antioxidant properties. The findings also suggest that fruit and vegetable wastes and by-products are good sources of natural bioactive compounds and these active compounds could be anticipated for further utilization in the food industries that will ultimately provide an economic and environmental impact.

In Chapter 3, antimicrobial properties of hot-water extracts from fruit wastes and by-products were assessed. The samples of extracts showed bacteriostatic as well as bactericidal effects against both the Gram-positive and -negative bacteria. The

remarkable antimicrobial effects were exhibited by the grape bunch stem extracts followed by grape wine pomace, Chinese quince sake pomace, immature peach and prune, irrespective of exposure time, doses, thermal stress and under acidic pH condition, whereas Gram-positive bacteria were more susceptible than the -negative. Thus, crude extracts from fruit wastes and by-products could be a potential source of antimicrobial candidacy.

In Chapter 4, antimicrobial effects of chlorogenic acid (CGA) and related compounds were measured. CGA and related compounds have shown specific antimicrobial activities and corresponding reduction in log survival ratio, whereas bactericidal effects were associated with treatment time, temperature, and doses. Notably, log survival ratio was mediated by pH and under thermal stress condition. It was demonstrated that CGA and related compounds exhibited a potent antimicrobial activities with the synergistic effects. Thus, we propose that CGA and related compounds could be useful as antimicrobial agents for food safety and hygiene during packaging and/or storage.

In Chapter 5, generally the results show that the functional food components of agricultural wastes and by-products having antioxidant as well as antimicrobial potency. In addition, the modes of action of antimicrobial activities depend on the type of

microorganisms and their cell wall structure, whereas the bioactive compounds of these extracts have shown more efficacy against Gram-positive bacteria than the -negative.

In conclusion, fruit and vegetable wastes and by-products having natural bioactive components that revealed antioxidant as well as antimicrobial properties against various microorganisms. Further, utilization of functional food components from these extracts will improve agricultural sustainability by maximizing the use of agricultural wastes and by-products.

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CHAPTER I

BACKGROUND AND PERSPECTIVES

A variety of agricultural wastes and by-products has been generating during food processing and cultivation. Most of the time these wastes and by-products are unutilized and may causes adverse environmental changes. It has been assumed that underutilized agricultural wastes and by-products carry plenty of functional food components. To investigate the nutraceutical of agricultural wastes and by-products and their utilization is ongoing demand. Food is the ideal vehicle for the dispersion of harmful agents which can cause life threatening food-borne illnesses. There are more than 80,000 chemicals and hundreds of naturally occurring biological pathogens, toxins, heavy metals, parasites that can cause serious illnesses. Food and food products are easily accessible at multiple points in any manufacturing process while they are easily distributed over great distances resulting in a great deal of concern for widespread impact of food-borne diseases. Food-borne diseases are an increasingly serious public health problem all over the world. The control of pathogens may significantly reduce the food-borne diseases outbreaks (1). In recent years, polyphenols, the secondary plant metabolites, have received a great deal of attention due to their diverse biological functions.

A considerable weight of evidence has been gathered suggesting that consumption of fruits and vegetables are beneficial for human health by the prevention of chronic diseases due to high value phenolic compounds (2). Likewise, some natural substances have antimicrobial properties (3). Spices and aromatic vegetable materials have long been used in food not only for their flavor and fragrance qualities and appetizing effects but also for their preservative and medicinal properties. It has been reported that the essential oils of spices have shown antimicrobial functions against food-borne pathogens (4). In addition, polyphenols may show beneficial biological properties, such as antimicrobial and antioxidant activities (5-7). Recently, the use of plants drugs are accepted all over the world. About 57% of the top-selling prescription in the USA contains natural products or derivatives, and one out of three Americans consumes herbal drugs (8,9). In developing countries, the use of medicinal plants has significantly increased due to the low income of the population. About 80% of the people are dependent, wholly or partially, on plant-based drugs. Herbs and spices are generally considered to be safe and proved to be effective against certain ailments. In recent years, use of spices/herbs has been gradually increasing (10) since a number of studies linked the high consumption of vegetable and fruits to the prevention of chronic diseases (11).

Interest in the antimicrobial properties of active compounds is strengthened by the findings that they affect the behavior of pathogenic bacteria or fungi of agro-food or medical field. Indeed, their use as natural additives in food industry is increased in recent years (12). The mechanisms responsible for phenolic toxicity to microorganisms include: adsorption and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation (13). Phenolic compounds can affect the growth and metabolism of bacteria, activating or inhibiting the microbial growth according to their constitution and concentration (12,14). Thus, we made an attempt to investigate the antioxidant, cytoprotective as well as antimicrobial activities of bioactive components from fruit and vegetable wastes and by-products as well as pure polyphenols.

OBJECTIVES OF THE STUDY

Throughout the world an unrestricted amount of agricultural wastes and by-products has been continuously producing during food processing and cultivation, which may contain potential source of bioactive components and nutraceutical. These wastes and by-products are generally unutilized and thrown away, that may causes adverse environmental changes and might be harmful for human health. The aim of this study was utilization of agricultural wastes and by-products. The experiments were conducted and the following parameters were studied to ascertain the above objectives:

- To investigate the functional properties of underutilized food components.
- To evaluate the antioxidant and cytoprotective activities of naturally occurring bioactive components of fruit and vegetable wastes and by-products.
- To examine the antimicrobial activities of nutraceutical from fruit wastes and by-products and also pure polyphenols against Gram-positive and -negative bacteria.
- To explore bioactive components prepared from agricultural wastes and by-products that might be effective to prevent or control contamination of food-borne pathogens and ultimately provide beneficial impact on human health.

CONSTRUCTION OF THE THESIS

Chapter I: Background and Perspectives as well as Objectives of the Study.

Chapter II: Antioxidant and Cytoprotective Activities of Fruit and Vegetable Wastes and By-products.

Chapter III: Antimicrobial Activities of Fruit Wastes and By-products.

Chapter IV: Antimicrobial Effects of Chlorogenic Acid and Related Compounds.

Chapter V: General Discussion, Concluding Remarks, and Suggestion for Future Research.

CHAPTER II

Antioxidant and Cytoprotective Activities of Extracts Prepared from Fruit and Vegetable Wastes and By-products

ABSTRACT

In this study, fruit and vegetable wastes and by-products were tested for polyphenols content and their antioxidant activity. The highest content of polyphenols as assessed by Folin-Ciocalteu assay were the hot-water extract of grape seed, followed by ethanolic extract of buckwheat hull. The highest antioxidant activity measured by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) assay were also detected in the hot-water extract of grape seed, followed by ethanolic extract of immature prune. Most of samples showed protective effects against oxidative stress induced by 2, 2'-Azobis- (2-amidinopropane) dihydrochloride (AAPH) peroxyl radical generator in African monkey kidney (MA 104) cells. Samples containing high amount of phenolics (more than 30 mg ChAE/g) generally showed high antioxidant activity and protective effect against AAPH-induced oxidative stress. This study demonstrates that fruit and vegetable wastes and by-products are good sources of high amounts of phenolics with antioxidant properties.

INTRODUCTION

Polyphenols are common constituents of the human diet, with fruits and vegetables being the major dietary source of these bioactive compounds. The possible health benefits of polyphenols consumption have been suggested to derive from their antioxidant properties (15). Dietary antioxidants are indeed believed to play a vital role in the human body defense system, protecting, as in plants, against oxidative damage induced by Reactive Oxygen Species, which are known to be involved in the pathogenesis of aging and many degenerative diseases (16). Current evidence strongly supports a contribution of polyphenols to the prevention of several chronic degenerative diseases such as cancer, atherosclerosis and cardiovascular diseases, central nervous system disorders, as well as aging (17).

Fruit and vegetable production and processing generate substantial quantities of waste/by-products. It has been previously reported that wastes and by-products of fruits may be an abundant source of antioxidant polyphenols (18,19). At the present time, fruit and vegetable wastes and by-products are often discarded at the expense of the manufacturer. Use of these wastes and by-products as a source of polyphenols may be of considerable economic benefit to food manufacturer. In addition, the antioxidant and

cytoprotective activities of polyphenols in fruit and vegetable wastes and by-products are of utmost importance to substantiate their potential health benefits in human nutrition.

The aim of the present study was to measure the relative content of phenolics in extracts prepared from fruit and vegetable wastes and by-products as well as to evaluate their antioxidant properties and cytoprotective activities under the same condition. The contents of total phenolics in extracts prepared from fruit and vegetable wastes by-products were analyzed by Folin-Ciocalteu assay. Antioxidant activities of all samples were measured by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) assay. Furthermore, cytoprotective activities of extracts were evaluated by the inhibitory effects against oxidative stress induced by 2, 2'-Azobis- (2- amidinopropane) dihydrochloride (AAPH) peroxyl radical generator in MA 104 cells.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu phenol reagent, chlorogenic acid hemihydrate (3-caffeoylquinic acid hemihydrate), and AAPH were purchased from Wako Pure Chemical Industries (Osaka, Japan). DPPH and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Plant materials

The samples investigated were three under-utilized fruit: Chinese quince (*Pseudocydonia sinensis*), quince (*Cydonia oblonga*), and hardy kiwi (*Actinidia arguta*); six fruit wastes: immature apple (*Malus pumila*), immature peach (*Prunus persica*), immature prune (*Prunus domestica*), immature pear (*Pyrus pyrifolia*), grape skin (*Vitis labruscana*: *V. labrusca* × *V. vinifera*), and grape seed (*Vitis labruscana*: *V. labrusca* × *V. vinifera*); eight vegetable wastes: broccoli leaf (*Brassica oleracea* Italica group), broccoli stem (*Brassica oleracea* Italica group), asparagus stem (*Asparagus officinalis*), cabbage outer leaf (*Brassica oleracea* Capitata group), Chinese cabbage outer leaf (*Brassica rapa* Pekinensis group), lettuce outer leaf (*Lactuca sativa* Capitata group), sweet potato vein (*Ipomoea batatas*), and cornhusks (*Zea mays*); seven by-products:

persimmon peel (*Diospyros kaki*), apple pomace (*Malus pumila*), wine pomace (*Vitis spp.*), grape bunch stem (*Vitis vinifera*), Chinese quince pomace (*Pseudocydonia sinensis*), quince pomace (*Cydonia oblonga*), and perilla pomace (*Perilla frutescens*); four hull: cowpea hull (*Vigna unguiculata*), black azuki bean hull (*Vigna angularis*), lima bean hull (*Phaseolus lunatus*), and buckwheat hull (*Fagopyrum esculentum*). Fruit and vegetable waste and by-products were obtained from different agro-industries of Nagano prefecture, Japan.

Hot-water extraction

Hot-water extraction was performed as follows: each plant material (100 g) was put into 4 times its volumes of boiling water (400 mL) and boiled for 1-h in a flask equipped with a reflux condenser. The suspended solution was filtered using two layers of cheesecloth and a filter paper (ADVANTEC, No. 2, Tokyo, Japan), concentrated using a rotary evaporator, and then lyophilized (Hot-water extracts). Samples were re-dissolved using distilled water. In case of cowpea hull, the extraction was conducted in eight times its volumes of water (800 mL) because of very high water absorbability.

Ethanol extraction

Ethanol extraction was performed as follows: each plant material (100 g) was immersed in 4 times its volumes of ethanol (for wet materials) or 80% (v/v) ethanol (for dried materials) and after one week at room temperature, the ethanol solution was filtered using a filter paper (ADVANTEC, No. 2). The filtrate was concentrated using a rotary evaporator (below 40℃), then lyophilized (Ethanolic extracts). Samples were re-dissolved using distilled water. In case of persimmon peel and apple pomace, the extraction was conducted in a hot-80% (v/v) ethanol (boiled for 1-h) in a flask equipped with a reflux condenser. The other procedures were the same as described previously.

Determination of total phenolics content

Total phenolic concentrations were measured using the Folin-Ciocalteu assay (20). Briefly, 10 μL of sample or a standard solution of chlorogenic acid hemihydrate was added into a 96-well microplate, followed by 40 μL of distilled water and 50 μL of Folin-Ciocalteu reagent diluted with distilled water (1:7 v/v). After 5 min, 50 μL of 9% of sodium carbonate aqueous solution was added and the contents were mixed thoroughly. The mixtures were allowed to stand for 90 min at room temperature in the dark. The absorbance was measured at 590 nm using a Model 680 Microplate Reader

(Bio-Rad Laboratories, Hercules, CA, USA). The results (average of three replicates ± SD) were expressed as milligrammes of chlorogenic acid hemihydrate equivalent per gram (mg ChAE/g) of extracted sample.

Free radical scavenging activity (DPPH assay)

The free radical scavenging activity using DPPH reagent was measured using the method (21) with some modification. Briefly, 10 µL of sample solution with different concentration or phosphate buffer saline (PBS) were added into 96-well microplate, followed by 40 µL of 0.5 mM Tris-HCl (pH 7.2). The reaction was initiated by the addition of 100 µL/well of DPPH solution in ethanol or ethanol for the color control. The mixtures were allowed to stand for 30 min at room temperature in the dark. The absorbance was measured at 520 nm using a microplate reader. The results (average of three replicates ± SD) were expressed as the EC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%.

Cell culture

MA 104 cells, an African monkey kidney cells, purchased from the ATCC (American Type Culture Collection), were maintained at 37℃ in an incubator with a humidified atmosphere of 5% CO_2. The MA 104 cells were cultured in GIT medium (Wako Pure Chemical Industries) containing streptomycin (100 μg/mL) and penicillin (100 units/mL).

Cell viability

Cell viability was estimated using the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (22). MA104 cells were seeded in a 96-well plate at a density of 8.0 x 10^3 cells/well. At 2 or 3 days after plating, cells were treated with 100 μg/mL of sample extract. After incubation for 3 h, the samples were removed and cells were treated with 10 μL of 0.8 mM AAPH solution, and 80 μl of PBS for 3 h. After removing AAPH solution, the cells were incubated for an additional 3 days and then treated with 70 μL of MTT solution containing 0.5 mg/mL of MTT in Eagle's minimum essential medium (Gibco, Grand Island, NY, USA) for 50 min at 37℃. The samples were then extracted with 90 μL of 0.04 N HCl-isopropanol and the absorbance was measured at 570 nm. The relative cell

viability was determined by the amount of MTT converted into the insoluble formazan salt. The optical density of the formazan formed in the untreated control cells was taken as 100% viability. Data are mean percentages of viable cells relative to the respective controls.

Statistical analysis

Statistical significance between groups were analyzed using Student's *t* test. *P* values < 0.05 were considered statistically significant. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Total phenolic content in extracts of fruit and vegetable wastes and by-products

The total phenolic content of individual samples were evaluated by Folin-Ciocalteu assay and the results are summarized in Table 2.1. The highest content in the extracted samples were the hot-water extracts of grape seed (43.9 ± 0.3 mg ChAE/g), followed by the ethanolic extract of buckwheat hull (38.0 ± 0.1 mg ChAE/g), whereas the lowest content was the ethanolic extract of asparagus stem (6.4 ± 0.0 mg ChAE/g). The phenolic compounds from grape seeds reported by previous study were gallic acid, (+)-catechin, (-)-epicatechin, the dimeric procyanidins B1 and B2, the trimeric procyanidin C1 and epicatechin gallate (23). Buckwheat hulls contain various polyphenols such as rutin, orientin, vitexin, quercetin, isovitexin and isoorientin (24).

There were some differences in the contents of total phenolics between hot-water extract and ethanolic extract. For example, the content of the hot-water extract of sweet potato vein (28.5 ± 0.3 mg ChAE/g) was higher than that of the ethanolic extract (14.9 ± .1 mg ChAE/g), while in the case of cowpea hull, the phenolic content of the hot-water extract (9.6 ± 0.1 mg ChAE/g) was lower than that of ethanolic extract (21.3 ± 0.2 mg ChAE/g). These data suggest that the extracted compounds differ in the extraction methods. Therefore, it is important to select the best way to extract efficiently.

Table 2.1 Total phenolic content (TPC) and DPPH radical-scavenging activity of hot-water and ethanolic extracts prepared from fruit and vegetable wastes and by-products

Parameter	Specimens	TPC[a]				DPPH[b]		
		Hot-water	Ethanol	*P* -value[c]		Hot-water	Ethanol	*P* -value[c]
Under-utilized fruits	Chinese quince	37.8±0.1	np	-		62.0±0.2	np	-
	Quince	18.4±0.2	np	-		356.4±11.7	310.4±10.8	0.0075
	Hardy kiwi	23.3±0.1	22.0±0.2	0.0005		105.7±13.1	233.1±8.2	0.0001
Fruits wastes	Immature apple	34.5±0.1	35.9±0.1	<0.0001		99.4±21.1	109.5±9.0	0.0025
	Immature peach	29.2±0.2	27.4±0.3	0.001		219.4±4.5	254.7±7.7	0.0024
	Immature prune	31.0±0.2	31.3±0.2	0.1401		160.2±1.6	15.2±0.5	<0.0001
	Immature pear	34.5±0.3	37.3±0.4	0.0006		106.7±2.3	122.8±1.8	0.0007
	Grape skin	10.3±0.1	np	-		338.3±9.8	np	-
	Grape seed	43.9±0.3	np	-		10.5±0.4	np	-
Vegetable wastes	Broccoli leaf	25.5±0.2	23.5±0.1	<0.0001		430.8±17.5	476.1±12.9	0.0227
	Broccoli stem	10.7±0.1	9.4±0.1	<0.0001		875.4±25.9	1319.5±50.5	0.0002
	Asparagus stem	7.3±0.0	6.4±0.0	<0.0001		2591.3±118.2	7097.6±795.7	0.0006
	Cabbage outer leaf	22.2±0.3	22.1±0.3	0.704		437.0±23.8	914.8±31.5	<0.0001
	Chinese cabbage outer leaf	16.1±0.2	19.0±0.3	0.0002		838.7±25.7	1085.0±28.5	0.0004
	Lettuce outer leaf	18.8±0.2	12.6±0.2	<0.0001		691.1±35.2	455.0±13.1	0.0004
	Sweet potato vein	28.5±0.3	14.9±0.1	<0.0001		np	np	-
	Corn husks	27.1±0.4	np	-		2062.0±32.4	np	-
By-products	Persimmon peel	17.0±0.1	16.7±0.2	0.0808		234.2±30.5	452.5±173.2	0.2213
	Apple pomace	11.6±0.1	14.5±0.1	<0.0001		331.0±294.2	195.0±157.0	0.6223
	Wine pomace	30.6±0.3	np	-		240.7±14.0	np	-
	Grape bunch stem	35.5±0.1	34.7±0.2	0.0034		127.5±0.9	203.0±19.2	0.0024
	Chinese quince pomace	13.3±0.3	7.3±0.1	<0.0001		898.0±27.3	2409.4±265.9	0.0006
	Quince pomace	7.6±0.1	np	-		2642.1±61.1	np	-
	Perilla pomace	32.9±0.4	np	-		297.7±76.0	np	-
Hull	Cowpea hull	9.6±0.1	21.3±0.2	<0.0001		2648.2±124.8	717.3±38.0	<0.0001
	Black azuki bean hull	16.8±0.2	np	-		1131.6±16.1	np	-
	Lima bean hull	23.1±0.1	33.1±0.1	<0.0001		np	np	-
	Buckwheat hull	37.3±0.2	38.0±0.1	0.001		80.9±1.5	37.6±6.7	0.0004

[a]Values expressed as mg chlorogenic acid hemihydrate equivalent/g (mg ChAE) dry sample (mean ± SD). [b]Values expressed as EC_{50}, concentration (μg/mL) at 50% scavenging of DPPH radicals. [c]Statistical differences between hot-water and ethanolic extracts were analyzed using Student's *t* test. [np]Not performed.

Free radical scavenging efficiencies of extracts from wastes and by-products against DPPH

Phenolics, commonly found in fruits, have been reported to exhibit antioxidant activity, due to the reactivity of the phenol moiety, and have the ability to scavenge free radicals (25). A causative relationship has been demonstrated between total phenolic content and antioxidant activity (26). The free radical scavenging efficiencies of extracted samples were measured by DPPH system. EC_{50} of the hot-water extract of grape seed was 10.5 ± 0.4 μg/mL and had the highest activity amongst of all samples, followed by the ethanolic extract of immature prune (15.2 ± 0.5 μg/mL), and the ethanolic extract of buckwheat hull (37.6 ± 6.7 μg/mL) (Table 2.1). Samples containing high amount of phenolics (more than 30 mg ChAE/g) generally showed a lower EC_{50}. However, the phenolic content of some samples were not correlated with EC_{50} measured by the DPPH system, such as the hot-water extracts of corn husks and cowpea hull. The EC_{50} of these samples were much higher than other samples. The relationships between particular antioxidants and antioxidant activity are difficult to be explained on the basis of quantitative analysis only. Not only the level of antioxidants but also an interactions occurring among them and other constituents might influence on the antioxidant activity (27).

Protective effect of extracts from wastes and by-products on AAPH-induced cytotoxicity in MA104 cells

Intracellular oxidation and cytotoxicity of MA 104 cells were induced with exposure to AAPH peroxyl radical generator. AAPH undergoes spontaneous thermal decomposition at 37℃ to produce two carbon-centered radicals that form peroxyl radicals upon exposure to oxygen (28). The role of extracts from fruit and vegetable wastes and by-products in the protection of the MA 104 cells from AAPH-induced oxidative stress were evaluated using the MTT assay (Fig. 2.1). The cells treated with 0.8 mM of AAPH without of any extract pretreatments showed the 76.0±0.9% of cell viability, when untreated control cells were taken as 100% viability. This AAPH concentration was used to investigate the protective effect of samples against AAPH-induced oxidative stress. As a result, all samples except the ethanolic extract of apple pomace and Chinese quince pomace, and the hot-water extract of quince pomace, showed protective effects and significantly inhibited cell death.

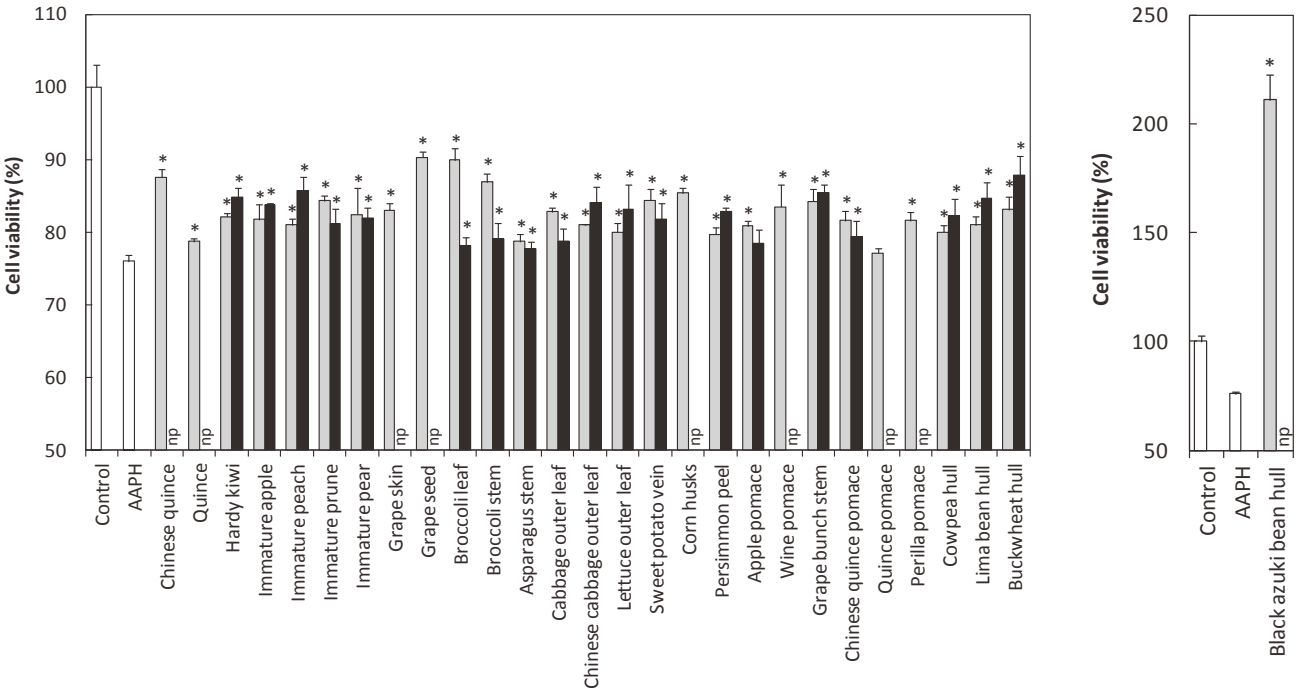

Figure 2.1 Effects of extracts from fruit and vegetable wastes and by-products on MA104 cell viability. MA 104 cells were treated with samples for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of viable cells in the untreated control. Data were expressed as mean ± SD (n=6). Asterisks (*) mean $p < 0.05$ and significant differences against the data of cells treated with AAPH alone. Control, □; AAPH, □; hot-water, ▒; ethanol, ■; not performed, np.

The hot-water extract of grape seed, which has high phenolic content and antioxidant activity, showed the high cytoprotective activity (89.9 ± 1.6%). The cell viabilities of the hot-water extracts of grape seed and broccoli leaf, and the ethanolic extract of buckwheat hull were relatively higher compared with other extracts. The low cytoprotective activity in the extracts of apple, Chinese quince, and quince pomaces were also low level of phenolic content and free radical scavenging efficiency. Surprisingly, the hot-water extract of black azuki bean hull showed 210.9 ± 11.5% of cell viability. This result suggests that some compounds in black azuki bean hull induce the cell proliferation. Previous study has showed that the extract of black azuki bean exhibited the strong antiproliferative properties in a dose-dependent manner against all digestive system cancer cell lines (CAL27, AGS, HepG2, SW480, and Caco-2), ovary cancer cell SK-OV-3 and breast cancer cell MCF-7 (29). Further studies are needed to identify the compounds in black azuki bean hull to induce cell proliferation, and the mechanisms of cell growth signaling.

In the present study, a total of 28 fruit and vegetable wastes and by-products were examined for total phenolics content as well as their antioxidant activity. Most extracts of fruit and vegetable wastes and by-products exhibited potent antioxidant activities in DPPH free radicals and AAPH peroxyl radicals. It was indicated that the highest level of antioxidants were detected in the extracts of grape seed, in addition immature prune and buckwheat hull exhibited efficient antioxidant activity with DPPH free radicals. A correlation was observed between antioxidant activities and phenolics content of extracts. Thus, fruit and vegetable wastes and by-products are the potential source of natural antioxidants. The exploitation of these abundant and low-cost renewable resources could be anticipated for the food industries during packaging and/or storage. This approach could ultimately provide a positive economic and environmental impact. Additional studies are needed for characterization of active compounds and the biological activities of these active extracts that might be included in nutraceutical formulations.

CHAPTER III

Antimicrobial Activities of Crude Extracts from Fruit Wastes and By-products

ABSTRACT

Antimicrobial effects of extracts from various underutilized food components were assessed using grape (*Vitis vinifera* L.) bunch stem, immature prune (*Prunus domestica* L.), immature peach (*Prunus persica* L.), Chinese quince (*Pseudocydonia sinensis* Schneid.) sake pomace, and grape (*Vitis vinifera* L.) wine pomace. Hot-water crude extracts were prepared from fruit wastes and by-products and assessed for their antimicrobial activities against *Escherichia coli* IFO 3301 and *Staphylococcus aureus* IFO 12732 using agar plate count and spectrophotometric assays. All extracts showed bacteriostatic as well as bactericidal effects against both the Gram-positive and -negative bacteria. A moderate growth inhibition and bactericidal effects were shown in immature prune and peach; in contrast, strong effects were observed in grape bunch stem followed by grape wine pomace and Chinese quince sake pomace extracts. Thus, crude extracts of fruit wastes and by-products are a potential source of antimicrobials.

INTRODUCTION

A large number of medicinal plants and underutilized food components have been the source of inspiration for novel drug compounds, as plant-derived medicines have been shown to be beneficial for human health (30). Extracts from different plants and underutilized food components may be useful in therapeutic applications against human pathogens (31). Bioactive components of fruits and vegetables have been shown to be beneficial for human health (2), including flavonoids, a major class of phytochemicals commonly found in fruits and vegetables (32). Considerable attention has been paid to polyphenols due to their diverse biological functions (33). For example, it is well known that phenolic compounds, as secondary plant metabolites, play a critical role in human health.

Epidemiological studies indicate that fruits, vegetables and plant-based phenolic metabolites are beneficial for humans due to their potent antioxidant activity and wide range of pharmacologic properties, such as anticancer, and platelet aggregation inhibitory activity (34-36). Both epidemiological and clinical studies have provided evidence that phenolic antioxidants present in cereals, fruits, and vegetables are principal contributing factors in the significantly reduced incidence of chronic and degenerative diseases in populations whose diet is high in these foods (37,38).

In the meanwhile, it has been reported that many plants extracts show to exert antimicrobial activity against various microorganisms (39). Further, the bioactive compounds of under-utilized wastes and by-products have also shown potential for their activities against various pathogens (40,41). To date, many researchers have reported that bacterial growth is affected by the constitution and concentration of phenolic compounds. Polyphenols have been shown to inhibit the growth of bacteria (14,42). Membrane permeability appears to be the major factor in the mechanism of antimicrobial action, resulting in membrane disruption, loss of cellular integrity and eventual cell death (43). Moreover, the mechanism of antimicrobial activity involves the adsorption to and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation (44,13).

In the context of the development of sustainable and eco-friendly agriculture, more active research on the health benefits of by-products and wastes continues to address an ongoing demand. Here, it was made an attempted to investigate the antimicrobial activities of extracts prepared from fruit wastes and by-products against Gram-positive and -negative bacteria.

MATERIALS AND METHODS

Materials

Grape (*Vitis vinifera)* bunch stem, (GST); immature prune (*Prunus domestica* L.), (IPR); immature peach (*Prunus persica* L.), (IPC); Chinese quince (*Pseudocydonia sinensis* Schneid.) sake pomace, (CQSP); and grape (*Vitis vinifera* L.) wine pomace, (GWP) were collected from Nagano Prefecture, Japan. Luria broth (LB) was purchased from Sigma-Aldrich (Tokyo, Japan). MacConkey and mannitol salt agar were from Nippon Seiyaku Co. Ltd. (Tokyo, Japan). *Escherichia coli* IFO 3301 and *Staphylococcus aureus* IFO 12732 were obtained from the Institute for Fermentation (Osaka, Japan). Other chemicals used were of biochemistry grade.

Preparation of crude extracts

Sample extracts were prepared according to the following hot-water extraction procedure. A portion (100 g) of the dried and powdered sample was weighed using an electronic balance, a 4-times volume of boiling water was added to the sample and boiled for 1-h in a flask equipped with a reflux condenser. After heating, all samples tested were centrifuged at 7,500 × g for 20 min, and the resulting supernatants were filtered with filter paper (Advantec, No. 2, Tokyo, Japan) to remove unutilized residues.

Next, the supernatant samples were lyophilized and stored in a refrigerator (-4℃) until used in experiments.

Determination of growth inhibitory activity

Bacterial cultures were prepared by overnight incubation in LB medium at 37℃ with shaking. The cultures were then washed two times and centrifuged 100 × g using PBS (phosphate buffer saline, pH 7). In addition, the tested bacterial cultures were diluted with PBS to give a concentration of 10^6 cells/mL, enumerated using a haematometer (Neubauer; LO-Laboroptik GmbH, Friedrichsdorf, Germany). A 10 μL aliquot of each overnight-cultured bacterial solution (*E. coli* IFO 3301 and *S. aureus* IFO 12732) was inoculated into 5 mL of LB medium. The final concentration of tested compounds in the LB medium was 1 mg/mL and a control (without sample) was taken to measure the bacterial growth inhibition. The cultures were incubated in a rotary shaker at 37℃ and the OD was measured at 600 nm wavelength using a UV-VIS spectrophotometer Shimadzu-1700 (Tokyo, Japan) to determine growth suppression. Spectrophotometry readings were taken at 1-h intervals for a total of 6 h. Growth curves were plotted using the obtained OD readings. The measurements were conducted in triplicate.

Determination of bactericidal effects

The bactericidal effects of tested samples were assessed using *E. coli* IFO 3301 and *S. aureus* IFO 12732. Log survival was measured using mid-logarithmic phase cell cultures (10^6 cells/mL) to identify the antimicrobial activity of tested compounds at different exposure times and concentrations, as well as thermal stress, under neutral and acidic pHs. First, the effects of exposure time on the bactericidal effects of tested compounds were measured. Bacterial cultures with 10 mg/mL test samples were incubated in a rotary shaker under neutral pH and 37℃ for 0, 1, 2, and 3 h time points to measure the log cells/mL. To measure the number of colony forming units (CFUs), a series of 10-fold dilutions were prepared using PBS (pH 7) and then plated onto MacConkey and mannitol salt agar plates. The plates were incubated at 37℃ for 24 h and the numbers of CFUs were enumerated, where the count detection limit was between 5 and 50 CFUs.

Secondly, the dose-dependent bactericidal effects of tested compounds were also assessed. The bactericidal effects of 3, 6, and 12 mg/mL test samples were measured using mid-logarithmic phase bacterial cultures (10^6 cells/mL) under 3 h incubation at 37℃ with shaking. The samples were then 10-fold diluted with PBS, spread on the

MacConkey and mannitol salt agar plates, and incubated at 37℃ for 24 h. After incubation, the numbers of viable colonies were determined as previously stated.

In addition, the role of thermal stress in the bactericidal effects of tested samples was assessed. Bacterial cultures (10^6 cells/mL) with 5 mg/mL test samples were incubated in a rotary shaker under neutral pH, at 37 and 45℃ for 3 h to measure the log survival ratio. The samples were then serially diluted, and cell suspensions were spread on MacConkey and mannitol salt agar plates and incubated for 24 h at 37℃. After incubation, the numbers of viable colonies were enumerated as previously stated.

Bactericidal effects under acidic pH

In addition to the above experiments, the bactericidal activity of test samples was measured under an acidic pH condition. Bacterial cultures at pH 7 and 5 were treated with 5 mg/mL samples, incubated in a rotary shaker at 37℃ for 3 h. PBS was used to prepare the buffer solutions at pH 7 and sodium acetate was used to prepare the buffer solutions at pH 5. After incubation, all cultures were 10-fold diluted with PBS; the decimal dilutions were spread on the MacConkey and mannitol salt agar plates and incubated at 37℃ for 24 h, and then viable colonies were determined as previously stated.

Statistical analysis

The results were expressed as the mean ± SD of three independent experiments. Statistical analysis was evaluated using a Student's *t*-test. Paired tests were used to assess differences between groups. Differences between means were calculated by one-way analysis of variance (ANOVA) following Duncan's new multiple range test (DMRT). A statistical probability of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Bacteriostatic effects of fruit wastes and by-products

The antimicrobial potential of hot-water extracts of grape bunch stem (GST), immature prune (IPR), immature peach (IPC), Chinese quince sake pomace (CQSP), and grape wine pomace (GWP) were tested against the Gram-positive bacterium *S. aureus* IFO 12732 and the Gram-negative bacterium *E. coli* IFO 3301. As shown in Fig. 3.1 & 3.2, crude extracts of the tested materials showed specific bacteriostatic effects and growth inhibitions at different time intervals against both of the bacteria. Fig. 3.1A shows the growth inhibitory effects of samples against *E. coli* under a neutral pH. The growth inhibitions were observed in the rank order of GST > GWP > CQSP > IPC > IPR. Among the samples, significant (p <0.05) growth inhibitions were shown by IPC, CQSP, GWP and GST (Fig. 3.1B). Further, significant and drastic bacteriostatic effects were revealed by CQSP, GWP, and GST. Fig. 3.2A shows the growth inhibitory effects against *S. aureus* under a neutral pH. The bacteriostatic effects were observed in the rank order of GST > GWP > CQSP > IPC > IPR. Significant (p <0.05) growth inhibitions were shown by IPC, CQSP, GWP, and GST (Fig. 3.2B). Further, remarkable and significant growth suppressions were observed in CQSP, GWP, and GST.

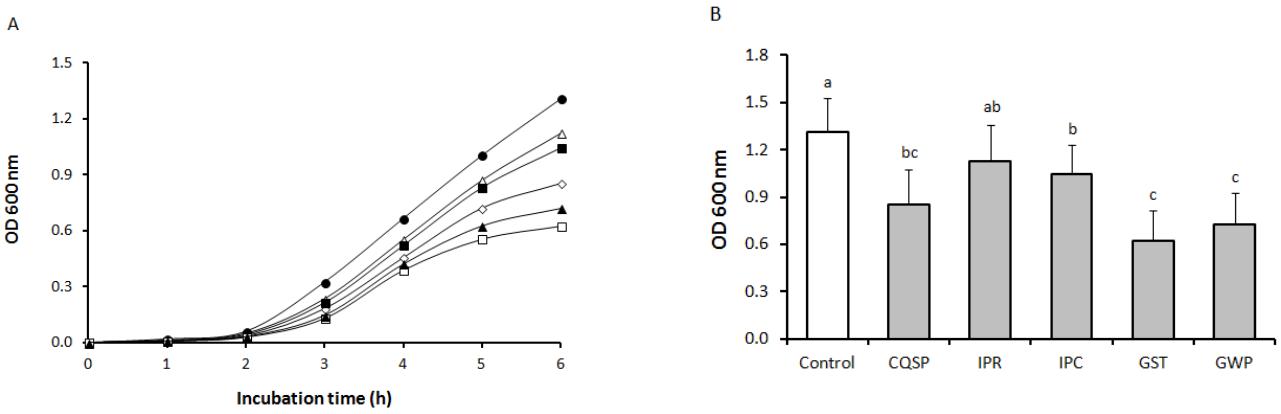

Figure 3.1 Growth inhibitory effects of fruit wastes and by-products against *E. coli* (A) under neutral pH, incubated at 37℃ for 6 h. Measurements at OD 600 nm were taken at 0, 1, 2, 3, 4, 5, and 6 h. B shows the growth inhibitory effects of wastes and by-products against *E. coli* after 6 h incubation, respectively. —●—, control; —◇—, CQSP; —△—, IPR; —■—, IPC; —□—, GST; —▲—, GWP. A shows representative data of three independent experiments. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences between means (p <0.05).

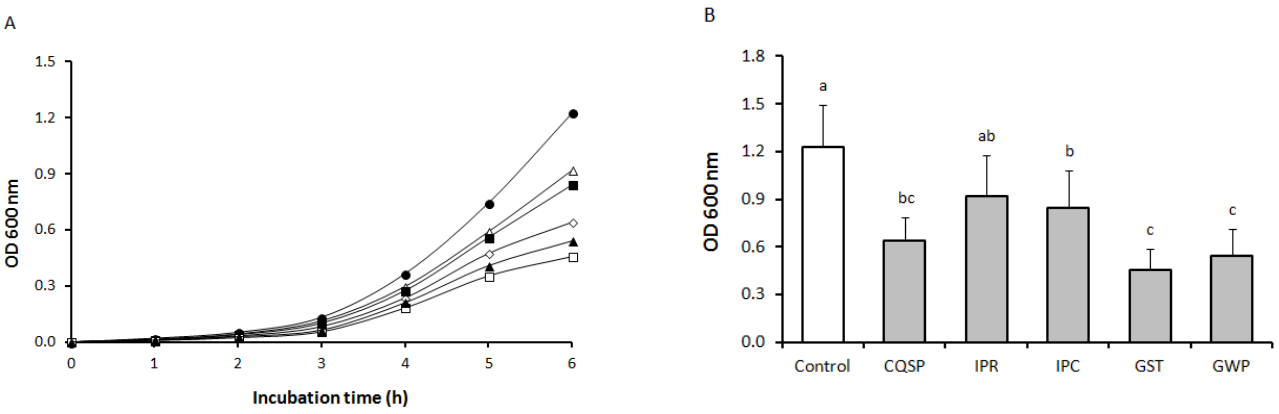

Figure 3.2 Growth inhibitory effects of fruit wastes and by-products against *S. aureus* (A) under neutral pH, incubated at 37℃ for 6 h. Measurements at OD 600 nm were taken at 0, 1, 2, 3, 4, 5, and 6 h. B shows the growth inhibitory effects of wastes and by-products against *S. aureus* after 6 h incubation, respectively. —●—, control; —◇—, CQSP; —△—, IPR; —■—, IPC; —□—, GST; —▲—, GWP. A shows representative data of three independent experiments. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences between means (p <0.05).

Phenolic compounds affect the growth and metabolism of bacteria, activating or inhibiting microbial growth according to their structure and concentration (12). It has been investigated that phenolic compounds extracted from under-utilized spices and herbs have shown potential antimicrobial activity against food-borne bacterial species, in which Gram-positive were more effective than -negative bacteria (45). Thus, it was confirmed the potent growth inhibitory effect of extracts prepared from fruit wastes and by-products.

Bactericidal effects of fruit wastes and by-products

- Effect of exposure time -

The effects of extracts exposure time were monitored against Gram-negative and -positive bacteria. All the tested samples showed specific exposure time-related lethal effects against *E. coli* under a neutral pH (Fig. 3.3A). Significant (p <0.05) bactericidal effects were observed in the rank order of GST > GWP > CQSP > IPC; an obvious bactericidal effect was indicated by GST (Fig. 3.3B). As shown in Fig. 3.4A, the effects of exposure time were demonstrated using *S. aureus* incubated at 37℃ under a neutral pH condition. Significant (p <0.05) bactericidal effects against *S. aureus* were shown by IPR, IPC, CQSP, and GWP, whereas no survival was observed for GST (Fig. 3.4B).

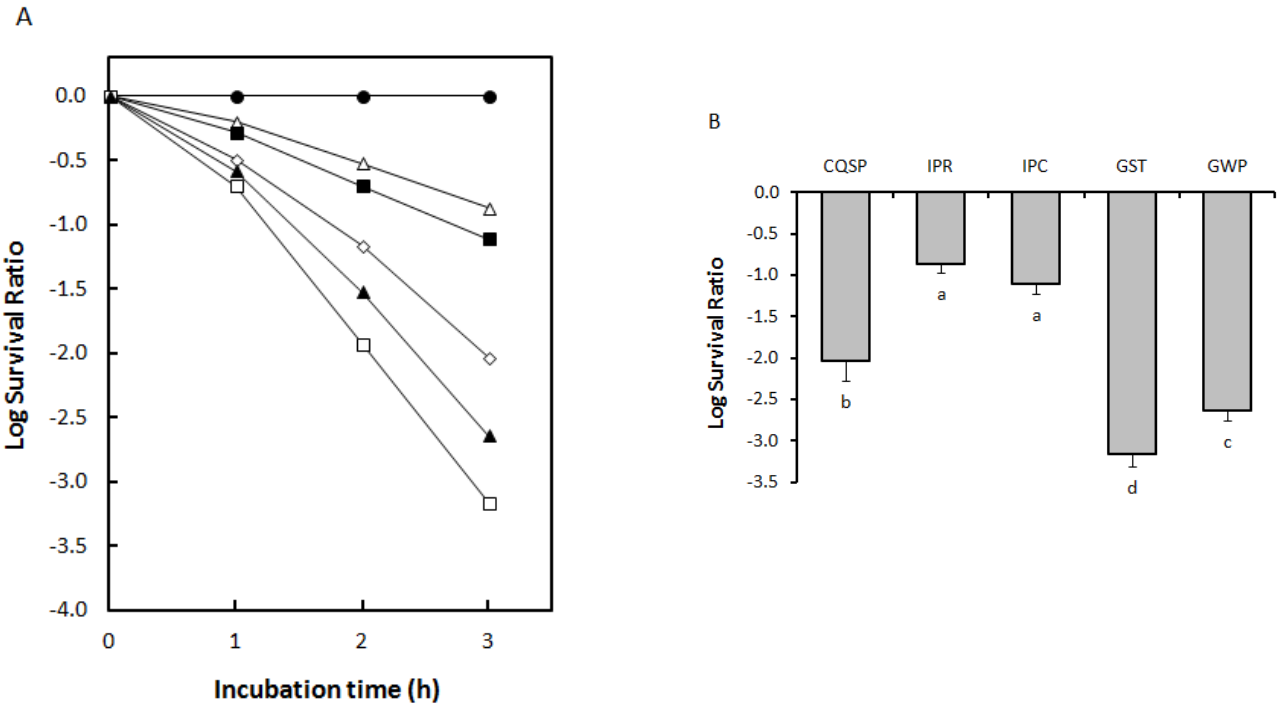

Figure 3.3 Effect of incubation time on the bactericidal effects of fruit wastes and by-products against *E. coli* (A) under neutral pH, incubated at 37℃ for 0, 1, 2, and 3 h. B shows the log survival ratio for *E. coli* exposed to wastes and by-products under neutral pH, incubated at 37℃ for 3 h, respectively. Log survival ratio was calculated by enumerating viable cells. —●—, control; —◇—, CQSP; —△—, IPR; —■—, IPC; —□—, GST; —▲—, GWP. Data shown in A represent three independent experiments. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences between means (p <0.05). NS, non-survival.

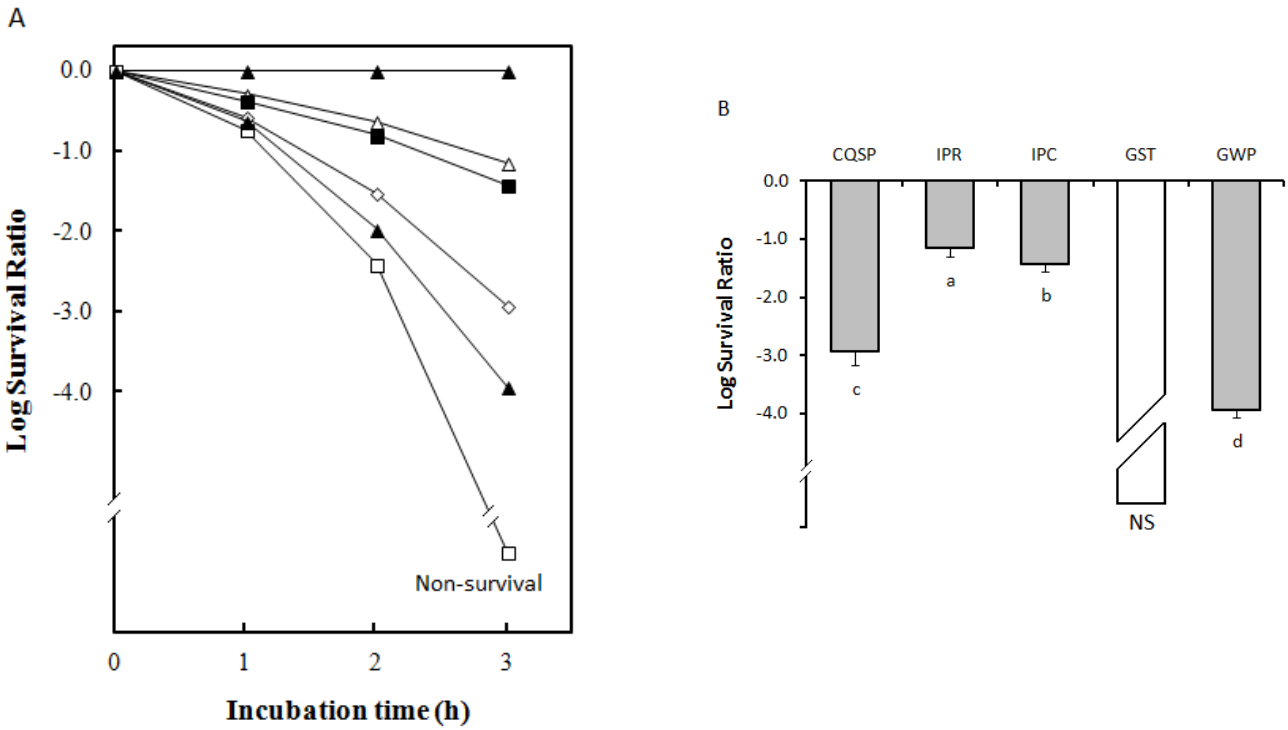

Figure 3.4 Effect of incubation time on the bactericidal effects of fruit wastes and by-products against *S. aureus* (A) under neutral pH, incubated at 37℃ for 0, 1, 2, and 3 h. B shows the log survival ratio for *S. aureus* exposed to wastes and by-products under neutral pH, incubated at 37℃ for 3 h, respectively. Log survival ratio was calculated by enumerating viable cells. —●—, control; —◇—, CQSP; —△—, IPR; —■—, IPC; —□—, GST; —▲—, GWP. Data shown in A represent three independent experiments. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences between means (p <0.05). NS, non-survival.

While similar lethal effects were observed for both Gram-positive and -negative bacteria, the bactericidal effects showed an increased tendency in Gram-positive bacteria. It was observed that the antimicrobial activity of the resveratrol was more effective against Gram-positive than -negative bacteria and the bactericidal effect of resveratrol was also reported to be associated with incubation time (46). Treatment time and bactericidal effects of phenolic compounds were observed to be related (47). It has been proposed that the mechanism of action of the antimicrobial activity is due to microbial cell wall disruption, leading to cell death (48). It was demonstrated that bactericidal effects of fruit wastes and by-products were mediated by the exposure time.

- Dose-dependency -

The dose-dependency of the bactericidal effects of extracts against *S. aureus* and *E. coli* were also investigated. Fig. 3.5 & 3.6 show the bactericidal effects of the crude extracts. Weak bactericidal effects were observed at 3 mg/mL, moderate effects at 6 mg/mL, and strong effects were shown at 12 mg/mL, indicating dose-dependent bactericidal effects against *E. coli* (Fig. 3.5A). Significant (p <0.05) bactericidal effects were found in the rank order of GST > GWP > CQSP > IPC > IPR; GST exhibited a remarkable bactericidal effect (Fig. 3.5B).

As shown in Fig. 3.6A, a similar dose-dependent lethal effect was observed against *S. aureus*. In addition, no survival was shown for 12 mg/mL of GWP or GST. Significant (p <0.05) lethal effects were observed with IPR, IPC, and CQSP; on the other hand, treatment with GWP and GST resulted in no survival (Fig. 3.6B). While similar dose-dependent bactericidal effects were observed against both Gram-positive and -negative bacteria, lethal effects were greater in Gram-positive bacteria than -negative bacteria. It was demonstrated that bioactive compounds of different plant extracts were more effective against Gram-positive than -negative bacteria (49). Notably, the antimicrobial activity of phenolic compounds was shown to be affected by the inactivation of intracellular enzymes, the rate of cellular penetration, and membrane permeability (43). The bactericidal effects of plant extracts against various microorganisms have also been reported in dose-dependent manner (50). A dose-dependent bactericidal effect was observed against grape seed extracts (51). In this research, it was found that the bactericidal effect of extracts from fruit wastes and by-products were exhibited in dose-dependent manner.

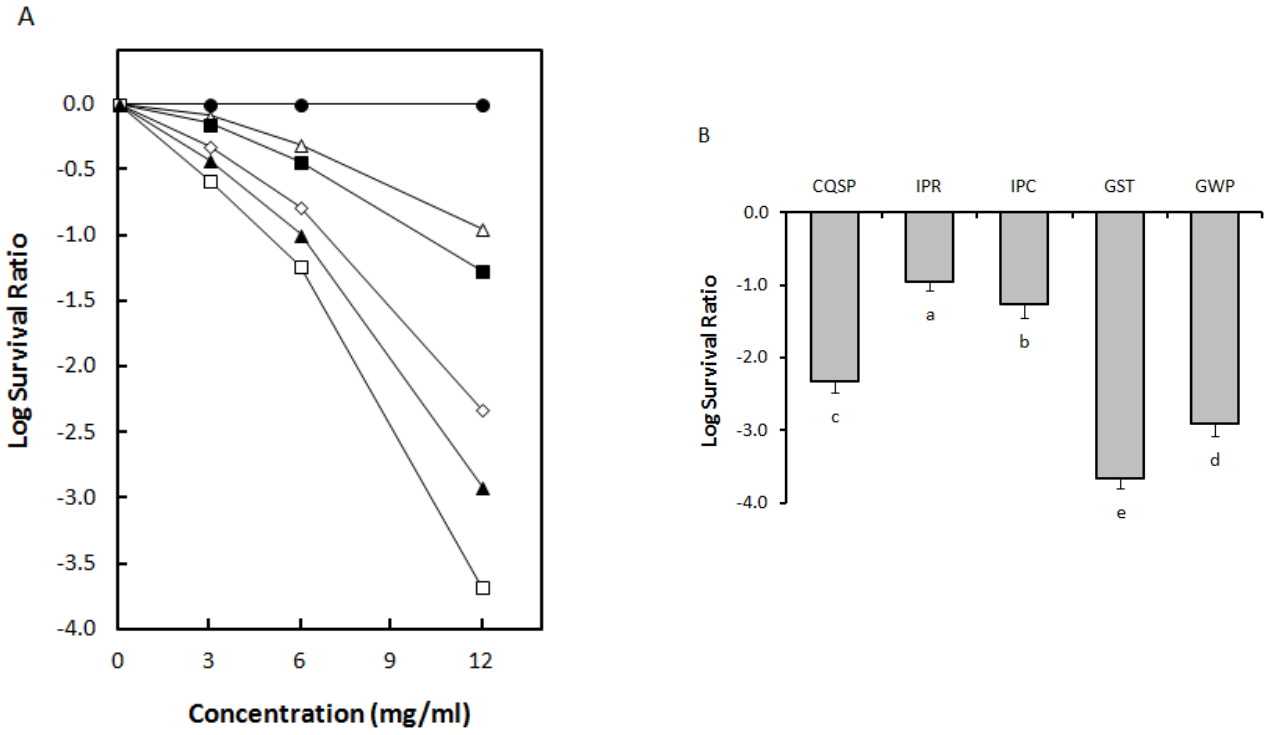

Figure 3.5 Effect of sample concentration on the bactericidal effects of fruit wastes and by-products against *E. coli* (A) under neutral pH, incubated at 37℃ for 3 h. B shows the log survival ratio for *E. coli* exposed to wastes and by-products (12 mg/mL) under neutral pH at 37℃ and 3 h incubation, respectively. —●—, control; —◇—, CQSP; —△—, IPR; —■—, IPC; —□—, GST; —▲—, GWP. A shows representative data of three independent experiments. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Log survival ratio was calculated by enumerating viable cells. Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences between means (p <0.05). NS, non-survival.

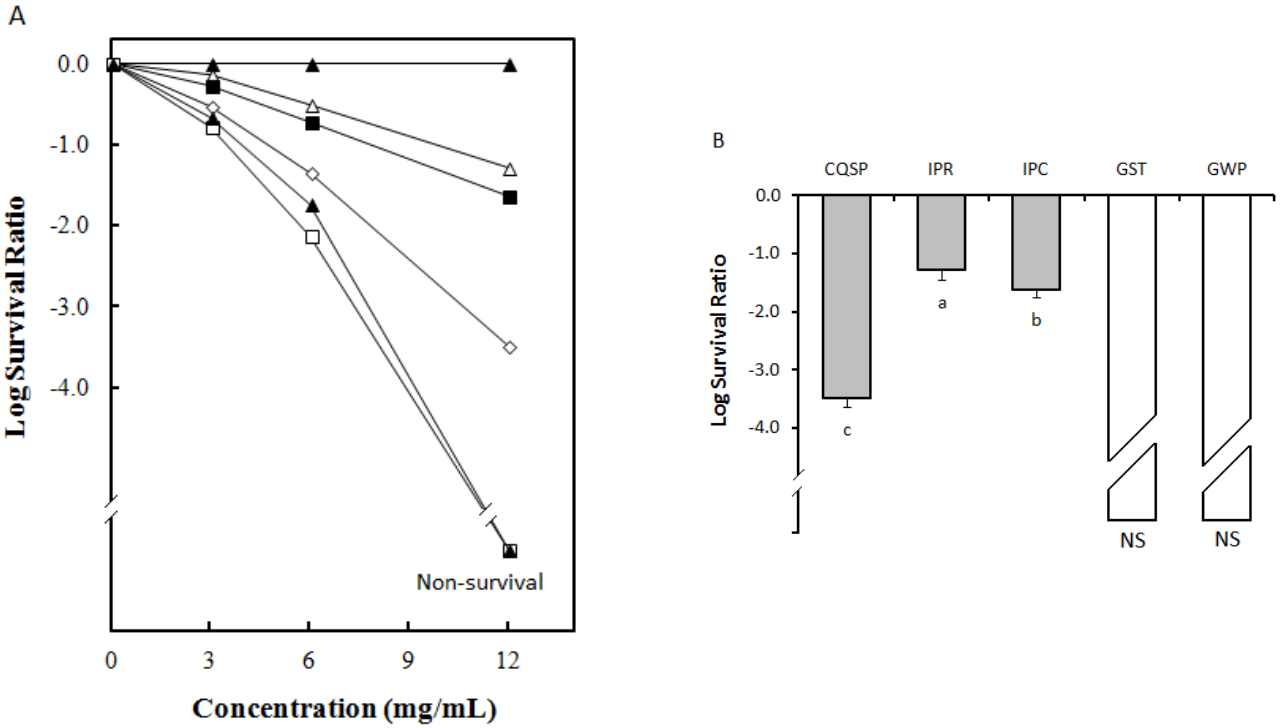

Figure 3.6 Effect of sample concentration on the bactericidal effects of fruit wastes and by-products against *S. aureus* (A) under neutral pH, incubated at 37℃ for 3 h. B shows the log survival ratio for *S. aureus* exposed to wastes and by-products (12 mg/mL) under neutral pH at 37℃ and 3 h incubation, respectively. —●—, control; —◇—, CQSP; —△—, IPR; —■—, IPC; —□—, GST; —▲—, GWP. A shows representative data of three independent experiments. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Log survival ratio was calculated by enumerating viable cells. Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences between means (p <0.05). NS, non-survival.

- Effect of thermal stress -

Bactericidal effects against *E. coli* and *S. aureus* under conditions of thermal stress and normal pH were assessed. As shown in Fig. 3.7, significant log reductions were observed under thermal stress condition. All extracts showed significant bactericidal effects against *E. coli* under thermal stress; specifically, a rapid and significant (p <0.01) bactericidal effects were found in CQSP, GWP, and GST (Fig. 3.7A). Significant bactericidal effects were also observed in the crude extracts of tested materials against *S. aureus*; specifically, a drastic and significant (p <0.01, p <0.001) log reductions were observed for CQSO, GWP, while GST treatment resulted in no survival (Fig. 3.7B). The results indicate that thermal stress plays a significant role in antimicrobial activities. The role of thermal stress in bactericidal effects was also reported (52). Furthermore, antimicrobial activity has been shown to be mediated by thermal or oxidative stress condition (53). Thus, it was confirmed that the bactericidal activity of extracts prepared from fruit wastes and by-products were regulated under thermal stress condition.

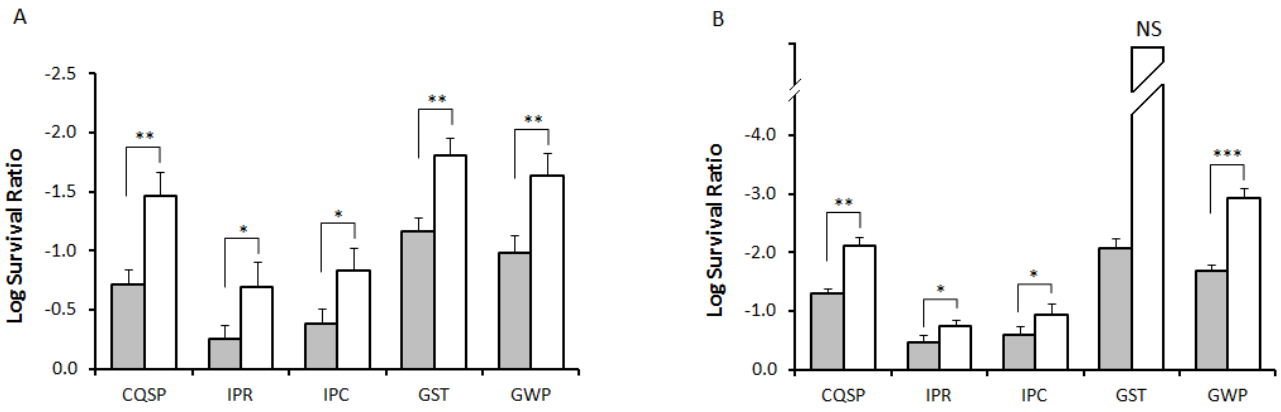

Figure 3.7 Bactericidal effects of fruit wastes and by-products against *E. coli* (A) and *S. aureus* (B) under neutral pH, incubated at (■) 37℃ and (□) 45℃ for 3 h. Log survival ratio was calculated by enumerating viable cells. CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace. Each value represents the mean ± SD of three independent experiments. Significant differences between means (*p <0.05, **p <0.01, ***p <0.001).

Bactericidal effects under acidic pH condition

The effects of pH on the antimicrobial potential of the tested compounds were also investigated against both of the Gram-positive and -negative bacteria. As shown in Table 3.1, the crude extracts of tested materials showed remarkable bactericidal effects against *S. aureus* and *E. coli* at different pHs. Significant (p <0.05) bactericidal effects were observed in GST as compared to other tested compounds against *E. coli* under a neutral pH condition, while significant lethal effects were shown for CQSP, GWP, and GST compared to the other samples against *E. coli* under an acidic pH condition. All samples showed significantly greater bactericidal effects against *E. coli* under an acidic pH condition than a neutral pH. Significant lethal effects were found under a neutral pH condition in the rank order of GST > GWP > CQSP > IPC against *S. aureus*, while under an acidic pH condition, significant lethal effects were observed in the rank order of CQSP > IPC, and no survivals were recorded for GWP and GST. Against *S. aureus,* all extracts showed significant lethal effects under an acidic pH condition as compared to a neutral pH, while no survivals were observed with GWP and GST. Interestingly, while notable bactericidal effects were observed against both Gram-positive and -negative bacteria under an acidic pH condition, Gram-positive bacteria were more susceptible than that of -negative bacteria to the extracts.

Table 3.1 Bactericidal effects of extracts from fruit wastes and by-products under neutral and acidic pH condition

Specimen	Log Survival (CFUs/mL)			
	Escherichia coli		*Staphylococcus aureus*	
	pH 7	pH 5	pH 7	pH 5
Control	6.03 ± 0.05^{a}	6.04 ± 0.09^{a}	6.05 ± 0.08^{a}	6.02 ± 0.07^{a}
CQSP	$5.29 \pm 0.13^{c**}$	$4.46 \pm 0.15^{c**}$	$4.71 \pm 0.10^{c***}$	$3.41 \pm 0.17^{c***}$
IPR	$5.75 \pm 0.12^{b*}$	$5.27 \pm 0.22^{b*}$	$5.54 \pm 0.13^{b*}$	$5.13 \pm 0.19^{b*}$
IPC	$5.62 \pm 0.14^{b*}$	$5.07 \pm 0.18^{b*}$	$5.40 \pm 0.15^{b*}$	$4.93 \pm 0.17^{b*}$
GST	$4.84 \pm 0.12^{d**}$	$3.92 \pm 0.14^{d**}$	3.93 ± 0.16^{e}	NS
GWP	$5.09 \pm 0.15^{cd**}$	$4.15 \pm 0.16^{d**}$	4.31 ± 0.11^{d}	NS

Bactericidal effects were tested at 37℃ and 3 h incubation under neutral and acidic pH condition. Control, without sample; CQSP, Chinese quince sake pomace; IPR, immature prune; IPC, immature peach; GST, grape bunch stem; GWP, grape wine pomace Each value represents the mean ± SD of three independent experiments. Different superscript letters indicate significant differences within columns (p <0.05). Significant differences between columns (*p <0.05, **p <0.01, ***p <0.001). NS, non-survival.

The antimicrobial activity of polyphenols is regulated by pH, causing physiological changes in microorganisms and eventual cell death (54). It was also demonstrated that the antimicrobial activity of phenolic compounds is mediated by pH, in which plant phenolic extracts were more effective against Gram-positive than -negative bacteria (55-57). In the present study, it was found that the bactericidal effect of extracts from fruit wastes and by-products were also mediated by pH.

The antimicrobial activities of crude extracts prepared from fruit wastes and by-products were assessed against both of Gram-positive and -negative bacteria. All extracts showed bactericidal as well as bacteriostatic effects against both the Gram-positive and -negative bacteria. The highest antimicrobial activities were detected for the extract of GST followed by GWP, CQSP, IPC, and IPR. In this study, it was confirmed that extracts prepared from fruit wastes and by-products showed potent antimicrobial activities irrespective of exposure time, dose, and conditions of thermal stress and acidic pH; further, Gram-positive bacteria showed greater susceptibility than the -negative bacteria. Thus, crude extracts from fruit wastes and by-products are potential candidates in developing antimicrobial agents to control food-borne pathogens during packaging and/or storage.

CHAPTER IV

Antimicrobial Effects of Chlorogenic Acid and Related Compounds

ABSTRACT

Chlorogenic acid (CGA) is a natural chemical ester composed of caffeic acid and (-)-quinic acid, and is further metabolized into active compounds in the living body. Here, it was aimed to provide fundamental information on the antimicrobial action of CGA and related compounds against the Gram-negative bacterium *Escherichia coli* IFO 3301. Bacteriostatic effects were assessed by spectrophotometry, and bactericidal effects were determined by enumerating viable cells on MacConkey agar plates. CGA and related compounds exhibited specific antimicrobial activity and corresponding reduction in log survival ratio, in which ferulic, isoferulic, benzoic, and hydroxybenzoic acids exhibited obvious antimicrobial activity against *E. coli*. In a time-kill assay, it was observed that bactericidal effects were associated with treatment time, temperature, and dose. Notably, a reduction in log survival ratio was observed at low pH as well as under thermal stress condition. Thus, it was demonstrated that CGA and related compounds have not only bacteriostatic but also bactericidal effects.

INTRODUCTION

Functional foods and bioactive components of fruits and vegetables are beneficial for human health (2). Polyphenols, which are secondary plant compounds, have received a great deal of attention due to their diverse biological functions (33). Among them, chlorogenic acid (CGA) holds promise as a physiologically active substance; its properties are attributable to the phenolic hydroxyl group(s) and it is characterized by relatively low toxicity and side effects. CGA is a natural phenolic compound commonly found in apples, coffee beans, grapes, pulp, peel, plum, and tea leaves (48, 58-60). It is well known that CGA possesses many health benefits, including antioxidative, chemopreventive, and other biological activities (12,61-63). To date, there have been several reports regarding the antimicrobial activity of phenolics, including flavonoids, in which the activity is derived from the disruption of microbial membranes, enzyme interaction, or metal ion deprivation (13,44,59). The antimicrobial activity of polyphenols has also been attributed to their structural features, as well as pH and sodium chloride concentration, resulting is physiological changes in the microorganisms and eventual cell death (54).

CGA is a phenolic ester of caffeic acid and (-)-quinic acid (64) and is metabolized to active compounds, such as quinic, caffeic, benzoic, hippuric, ferulic, isoferulic, and hydroxybenzoic acids, within the living body, (65-68); (shown in Fig. 4.1). Naturally occurring phenolic compounds in food have a potential as antioxidant, antibacterial, antifungal, and antiviral biomolecules (39,41,69-70). Unfortunately, to date, limited information is available on the antimicrobial effects of phenolic compounds and simultaneous parallel evaluation of these effects is lacking. Thus, it was attempted to assess the antimicrobial effects of CGA and related compounds using *Escherichia coli*, which is a typical Gram-negative bacterium that belongs to the most critical food-borne pathogenic bacteria.

MATERIALS AND METHODS

Materials

Dimethyl sulfoxide (DMSO), quinic acid, and ferulic acid were purchased from WAKO Pure Chemical Industries Ltd. (Tokyo, Japan). Luria broth (LB), and benzoic, hydroxybenzoic, caffeic, and hippuric acids were purchased from SIGMA-ALDRICH (Tokyo, Japan). MacConkey agar was purchased from Nippon Seiyaku Co., Ltd. (Tokyo, Japan). Isoferulic and chlorogenic acids were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *E. coli* IFO 3301 was purchased from the Institute for Fermentation (Osaka, Japan). All other chemicals used were of biochemical grade.

Culture conditions

E. coli IFO 3301 was incubated in LB medium at 37℃ overnight with shaking. After incubation, the bacterial culture was washed twice with phosphate buffered saline (PBS; pH 7.0) and centrifuged (1000 rpm). Next, the bacterial solution was adjusted with PBS to give a concentration of 10^6 cells/mL using a haematometer (Neubauer, LO-Laboroptik GmbH, Friedrichsdorf, Germany).

Microbial growth inhibition

A 10 μL aliquot of an overnight culture of *E. coli* was inoculated into 5 mL of LB medium. CGA and related compounds were added at a final concentration of 0.5 mM and growth inhibition was measured; control cultures were prepared without test samples. The cultures were incubated in a rotary shaker at 37℃ and the optical density (OD) at 600 nm was measured using a UV-VIS spectrophotometer SHIMADZU-1700 (Japan) to determine the growth inhibition. OD readings were taken for 36 h, at 0, 3, 6, 12, 18, 24, and 36 h time points. A growth curve was plotted with the values obtained from OD readings. All the measurements were done in triplicate.

Bactericidal effect

The bactericidal effects of CGA and related compounds were assessed using *E. coli* IFO 3301 as the test bacterium. Log survival was measured using mid-logarithmic phase cell cultures (10^6 cells/mL) to identify the antimicrobial action of CGA and related compounds at different doses, treatment times, temperature, and under acidic and neutral pH conditions.

Synergistic effect

The combined effect of CGA and related compound was measured. The bactericidal effects of 10 mM CGA and related compounds were measured with mid-logarithmic phase cell cultures on 6 h incubation at 37℃ with shaking. Then the samples were 10-fold diluted using PBS and incubated on agar plate at 37℃ for 18 h. After incubation visible colony appeared on plates were counted.

Dose-dependent effect

The dose-dependent bactericidal effects of CGA and related compounds were also measured. Bacterial cultures (10^6 cells/mL) with 2.5, 5.0, and 10 mM test samples were incubated in a rotary shaker at 37℃ for 6 h, and the log survival ratio was determined using agar plates.

Time-dependent effect

The effects of treatment time on the bactericidal effect of CGA and related compounds were also measured. Bacterial cultures with 5.0 mM test samples were incubated in a rotary shaker under neutral pH, at 37℃ and 50℃ for 0, 1, 3, and 6 h time points to measure the log cells/mL. After incubation, all cultures were microdiluted

using PBS; decimal dilutions of cell suspensions were spread on MacConkey agar plates and incubated at 37℃ for 18 h, and then viable cells were enumerated.

pH-dependent effect

The role of pH on the bactericidal effect of CGA and related compounds were assessed. Cultures at pH of 8.0, 7.0, 6.0, 5.0, and 4.0 were treated with 2.5 mM test samples, incubated in a rotary shaker at 37℃ for 3 h, and log survival was measured. PBS was used to prepare the buffer solutions at pH 8.0, 7.0 and 6.0, and sodium acetate was used to prepare the buffer solutions at pH 5.0, and 4.0.

Thermal stress

The role of thermal stress on the bactericidal effect of CGA and related compounds were assessed. Bacterial cultures (10^6 cells/mL) with 2.5 mM test samples were incubated in a rotary shaker under neutral pH, at 20, 37, 45, and 50℃ for 3 h to measure the log survival ratio.

To measure the number of colony forming units (CFUs) a serial 10-fold dilution were prepared using PBS (pH 7.0) and plated onto MacConkey agar plates. After incubation of the plates at 37℃ for 18 h, the numbers of CFUs were counted, and the limits of count detection were between 5 and 50 CFUs.

Statistical analysis

All of the experiments were performed in triplicate and the results were expressed as mean ± SD. Differences between means were assessed using analysis of variance (ANOVA) followed by Duncan's new multiple range test (DMRT). A statistical probability of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Bacteriostatic effects of CGA and related compounds

The antimicrobial effects of CGA and related compounds were investigated using a typical Gram-negative bacterium *E. coli*, because *E. coli* is the most critical, large and diverse group of Gram-negative food-borne pathogenic bacterium. Fig. 4.2A shows the specific bacteriostatic effect of CGA and related compounds against *E. coli*, where the growth inhibitory rate was measured at 600 nm under neutral pH. Comparatively lower growth inhibition was observed in CGA, quinic acid, and hippuric acid, and moderate activity was observed in caffeic acid. On the other hand, obvious growth inhibition was observed in hydroxybenzoic, benzoic, isoferulic, and ferulic acids. The Fig. 4.2B shows the growth inhibition of CGA and related compounds after 36 h incubation of cultures. As compared to control, significant growth inhibitions were shown in CGA and its related compounds. The bacteriostatic effect was in the rank order of quinic acid < chlorogenic acid < hippuric acid < caffeic acid < hydroxybenzoic acid < benzoic acid < isoferulic acid < ferulic acid. In contrast, potent and remarkable bacteriostatic effects were observed in ferulic, isoferulic, benzoic, and hydroxybenzoic acids compared to the other compounds. These results showed that CGA and related compounds possess a bacteriostatic effect that differs in efficacy.

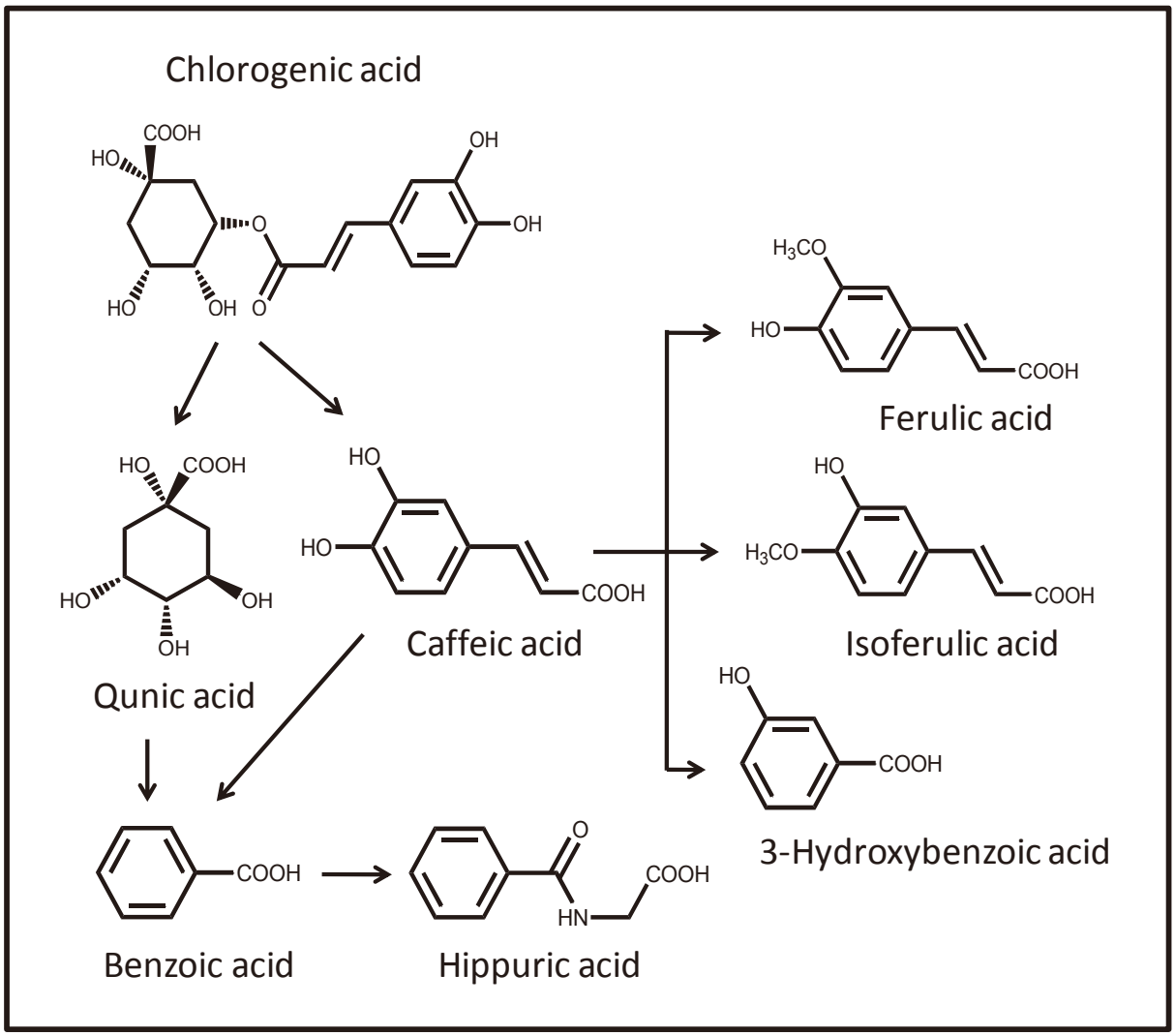

Figure 4.1 Chemical structures of CGA and related compounds. Arrows indicate the metabolic routes of CGA and related compounds as proposed by Gonthier et al. (2003).

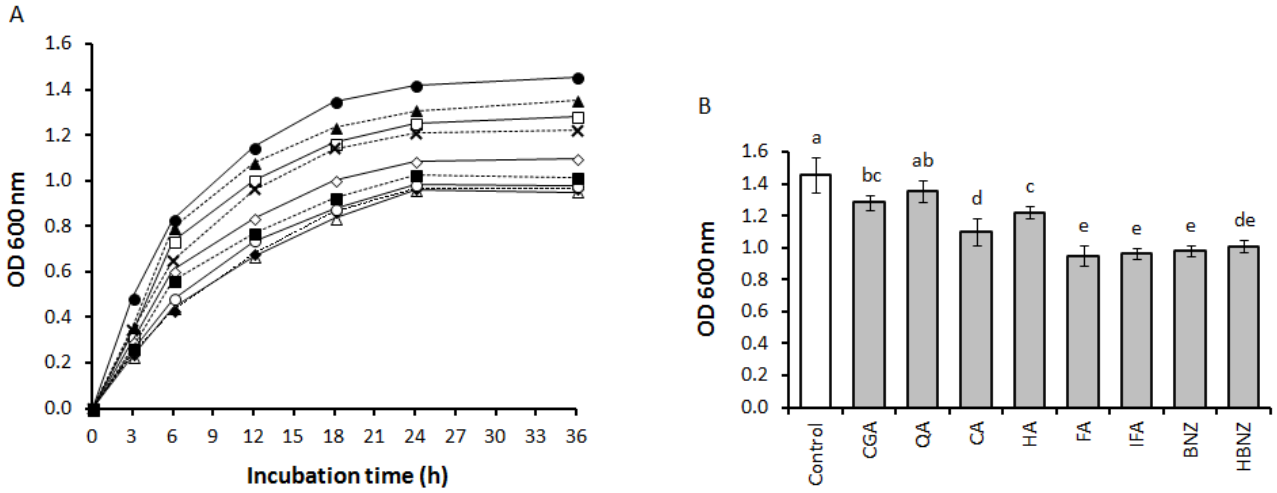

Figure 4.2 Growth inhibitory effects of CGA and related compounds against *E. coli* IFO 3301 under neutral pH, incubated at 37℃ for 36 h. Measurements at OD 600 nm were taken at 0, 3, 6, 12, 18, 24, and 36 h (A). The growth inhibition of CGA and related compounds after 36 h incubation of cultures (B). —●—, control; —□—, chlorogenic acid; ···▲···, quinic acid; —◇—, caffeic acid; ···×···, hippuric acid; —△—, ferulic acid; ···◆···, isoferulic acid; —○—, benzoic acid; ···■···, hydroxybenzoic acid; CGA, chlorogenic acid; QA, quinic acid; CA, caffeic acid; HA, hippuric acid; FA, ferulic acid; IFA, isoferulic acid; BNZ, benzoic acid; HBNZ, hydroxybenzoic acid. Each value represents the mean ± SD (n=3) of three independent experiments. Different superscripts represent significant differences between mean (p <0.05).

Table 4.1 Synergistic effect of caffeic acid and quinic acid on *E. coli* IFO 3301 survival

Tested Samples	Log Survival (CFUs/mL)
Control	6.00 ± 0.29^{a}
Chlorogenic acid	4.99 ± 0.25^{b}
Quinic acid	5.45 ± 0.39^{b}
Caffeic acid	4.30 ± 0.30^{c}
Quinic acid + caffeic acid	3.71 ± 0.23^{d}

The synergistic effect of CGA and related compounds was measured at 37℃ with 10 mM concentration after 6 h incubation under neutral pH with shaking. Each value represents mean ± SD (n=3). Different superscript letters indicate significant differences (p <0.05).

It has been demonstrated that polyphenols can inhibit the growth of bacteria, including *E. coli* (42,71). The structural features and concentration of the phenolic compounds play roles in the effects on bacterial growth and metabolism (12,14).

Bactericidal effects of CGA

CGA consists of quinic and caffeic acids, and readily breaks down into individual components (Fig. 4.1). Thus, it was investigated the bactericidal effects of CGA, quinic acid, and caffeic acid against *E. coli* under standard growth conditions (37℃ and pH 7.0; 50 mM PBS) with 6 h incubation. The result showed, no significant difference was observed between the bactericidal effects of chlorogenic and quinic acids, whereas caffeic acid significantly reduced CFUs, as shown in Table 4.1. Notably, the bactericidal activity of caffeic acid was improved in combination with quinic acid. In a time-kill assay, it was reported the synergistic action of phenylpropanoids (72). They proposed that CGA possesses a different antimicrobial action in the living body upon metabolism. Chlorogenic acid acts in synergy with other components to exhibit its full antimicrobial effect, and these mechanisms are thought to be responsible for the toxicity of phenolic compounds in microorganisms, including adsorption and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation (59).

Dose-dependency of log survival

The dose-dependent bactericidal effects of CGA and related compounds against *E. coli* were also investigated. Fig. 4.3A shows the bactericidal effect of different concentrations of CGA and related compounds against *E. coli* under neutral pH and 6 h incubation. A dose-dependent reduction in log survival ratio was observed for all compounds against *E. coli*. A relatively low bactericidal effect was observed in quinic acid, followed by chlorogenic acid < hippuric acid < caffeic acid < hydroxybenzoic acid < benzoic acids. On the other hand, a rapid lethal effect was shown in isoferulic and ferulic acids and moderate lethal effect was observed at a low concentration (2.5 mM). In contrast, a drastic reduction in log survival ratio was identified at a higher concentration (10 mM). It was revealed that CGA and related compounds showed a dose-dependent bactericidal effect against *E. coli*, as demonstrated against various microorganisms, including *E. coli* (50). Fig 4.3B shows the log survival ratio of CGA and related compounds at 37℃ with 10 mM concentration for 6 h incubation under neutral pH. No significant survivors were observed in between chlorogenic acid and hippuric acid.

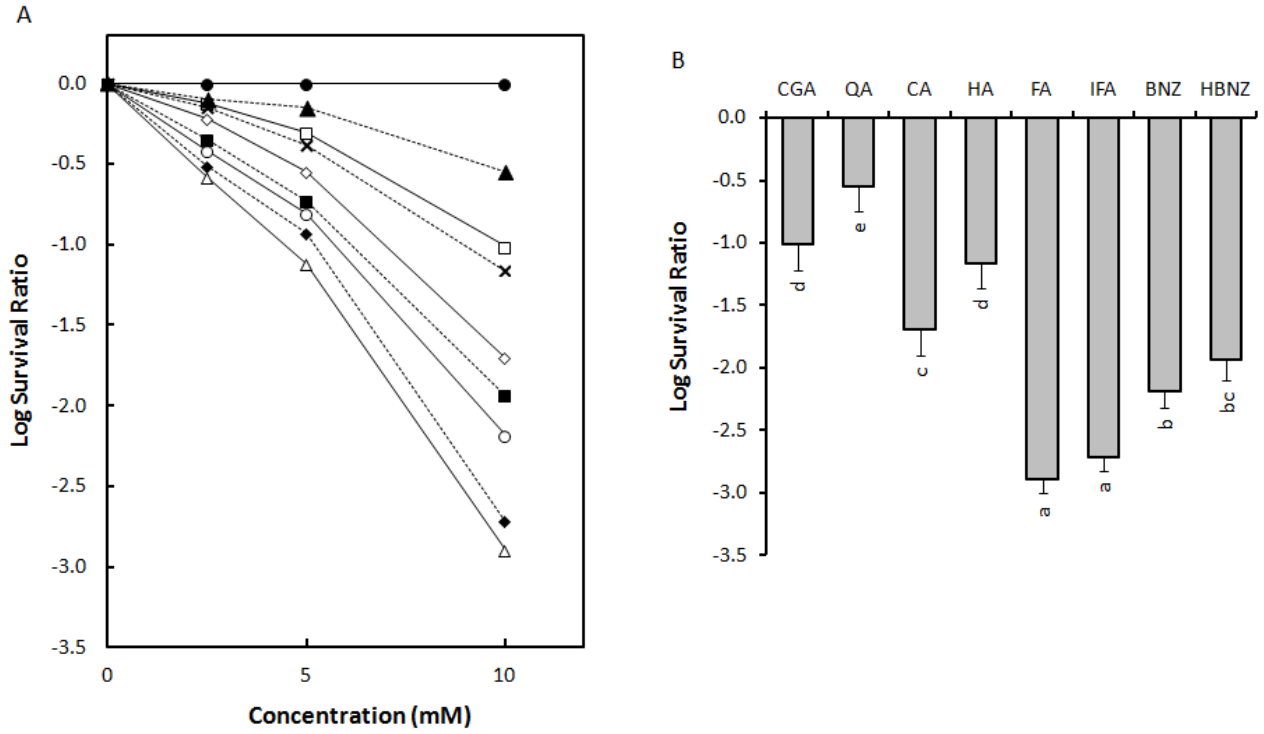

Figure 4.3 Effect of dose on the bactericidal activity of CGA and related compounds against *E. coli* IFO 3301 under neutral pH at 37℃ for 6 h (A). Log survival ratio was calculated by enumerating viable cells. Log survival ratio of CGA and related compounds was measures with 10 mM concentration for 6 h incubation (B). The data represent three independent experiments. —●—, control; —□—, chlorogenic acid; ···▲···, quinic acid; —◇—, caffeic acid; ···×···, hippuric acid; —△—, ferulic acid; ···◆···, isoferulic acid; —○—, benzoic acid; ···■···, hydroxybenzoic acid; CGA, chlorogenic acid; QA, quinic acid; CA, caffeic acid; HA, hippuric acid; FA, ferulic acid; IFA, isoferulic acid; BNZ, benzoic acid; HBNZ, hydroxybenzoic acid. Each value represents mean ± SD (n=3). Different superscripts represent significant differences between mean (p <0.05).

In contrast, a rapid log reduction was shown in caffeic acid followed by hydroxybenzoic acid < benzoic acid < isoferulic acid < ferulic acid. These results indicate that CGA and related compounds possess potent bactericidal action.

Effect of treatment time on log survival under different temperatures

Bacterial time-kill effects were also assessed. CGA and related compounds exhibited a treatment-time-dependent lethal effect against *E. coli* at 37℃ and neutral pH (Fig. 4.4A). The effect of treatment time on the bactericidal activity of samples was assessed. A comparatively higher lethal effect was observed after 6 h incubation. The bactericidal effect was in the rank order: ferulic acid > isoferulic acid > benzoic acid > hydroxybenzoic acid > caffeic acid > hippuric acid > chlorogenic acid > quinic acid. Depending on the treatment time, CGA and related compounds showed specific bactericidal effects under neutral pH against *E. coli* at 50℃, as shown in Fig. 4.4B. A relatively lower bactericidal effect was found after 1-h incubation and a moderate lethal effect was observed after 3 h incubation. In addition, significant bactericidal effects were revealed after 6 h exposure. After 6 h incubation, the lowest reduction in log survival ratio was with quinic acid, followed by chlorogenic, hippuric, caffeic, hydroxybenzoic, and benzoic acids.

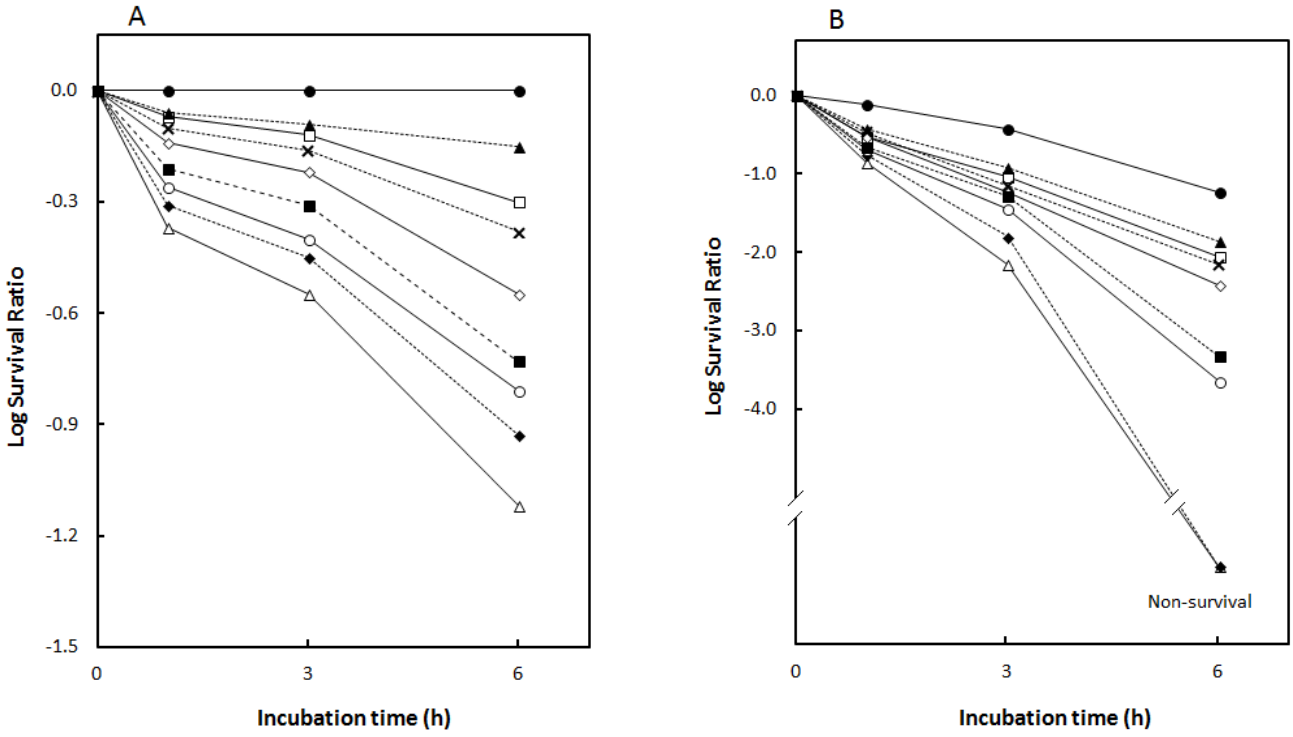

Figure 4.4 Effect of treatment time on the bactericidal activity of CGA and related compounds against *E. coli* IFO 3301 under neutral pH, incubated at (A) 37℃ and (B) 50℃ for 0, 1, 3, and 6 h. Log survival ratio was calculated by enumerating viable cells. Each value represents three independent experiments. —●—, control; —□—, chlorogenic acid; ···▲···, quinic acid; —◇—, caffeic acid; ···×···, hippuric acid; —△—, ferulic acid; ···◆···, isoferulic acid; —○—, benzoic acid; ···■···, hydroxybenzoic acid.

On the other hand, no survivability was shown in ferulic and isoferulic acids. This indicates that treatment times as well as temperature are the vital factors in the bactericidal effects. It was confirmed that CGA and related compounds possess potent treatment-time-temperature-dependent bactericidal effects. Here it was found significantly higher bactericidal effect under the same treatment time with higher temperature that may be due to higher penetration ability of CGA and related compounds against bacterial cell membrane, which indicate temperature mediated membrane permeability. The mechanism of action may be due to microbial cell wall disruption, leading to cell death (47). It was investigated that microbial killing effects of phenolic compounds were time-dependent (48). The antimicrobial activity of phenolic compounds is related to the inactivation of cellular enzymes, which are dependent on the rate of cellular penetration of a substance or affected by alterations in membrane permeability (43). Increased membrane permeability is a major factor in the mechanism of antimicrobial activity, where compounds disrupt membranes, leading to loss of cellular integrity and eventual cell death (43).

Effects of pH on log survival

The pH-dependent antimicrobial effect of CGA and related compounds was also investigated. As shown in Fig. 4.5, CGA and related compounds showed potent pH-dependent bactericidal effects against *E. coli* incubated at 37℃ for 3 h. A weak bactericidal effect was found at pH 8.0 as well as at 7.0. On the other hand, moderate reductions in log survival ratios were observed at pH 6.0, whereas a drastic lethal effect was revealed at pH 5.0 < pH 4.0. At both pH 6.0 and pH 5.0, the bactericidal effects of hydroxybenzoic, benzoic, ferulic, and isoferulic acids were comparatively higher than those of quinic, chlorogenic, hippuric, and caffeic acids. At pH 4.0, lethal effects were observed for CGA and all related compounds except quinic acid. Thus, pH appears to be a vital factor in the bactericidal action of CGA and related compounds. In this study, it was confirmed that CGA and related compounds exerted significant reductions in log survival ratio at different pHs. It was reported that the antimicrobial activity of phenolic compounds is mediated by pH (55,56).

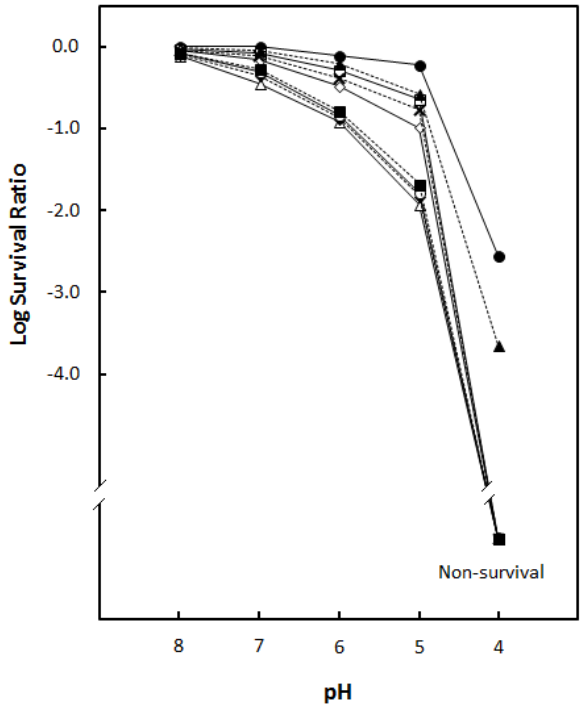

Figure 4.5 Effect of pH on the antimicrobial activity of CGA and related compounds against *E. coli* IFO 3301, incubated at 37℃ for 3 h. A range of pH (8.0, 7.0, 6.0, 5.0, and 4.0) was assessed. Log survival ratio was calculated by enumerating viable cells. The data represent three independent experiments. —●—, control; —□—, chlorogenic acid; ···▲···, quinic acid; —◇—, caffeic acid; ···×···, hippuric acid; —△—, ferulic acid; ···◆···, isoferulic acid; —○—, benzoic acid; ···■···, hydroxybenzoic acid.

Effects of thermal stress on log survival

CGA and related compounds exhibited lethal effects against *E. coli* under thermal stress with normal pH and 3 h incubation, as shown in Fig. 4.6. A weak bactericidal effect was observed at 20℃ and a rapid and marked bactericidal effect was found at 45℃; whereas at 37℃ a moderate log reduction was observed in quinic acid followed by chlorogenic, hippuric, and caffeic acids. In contrast, hydroxybenzoic, benzoic, ferulic, and isoferulic acids revealed greater bactericidal effects. At 50℃, quinic, chlorogenic, hippuric, caffeic, hydroxybenzoic, and benzoic acids showed moderate bactericidal effects, while ferulic and isoferulic acids showed enhanced bactericidal activity. It is proposed that the lethal effect of CGA and related compounds under thermal stress was predominantly associated with the antimicrobial activity, and the highest bactericidal action was identified in ferulic, isoferulic, benzoic, and hydroxybenzoic acids.

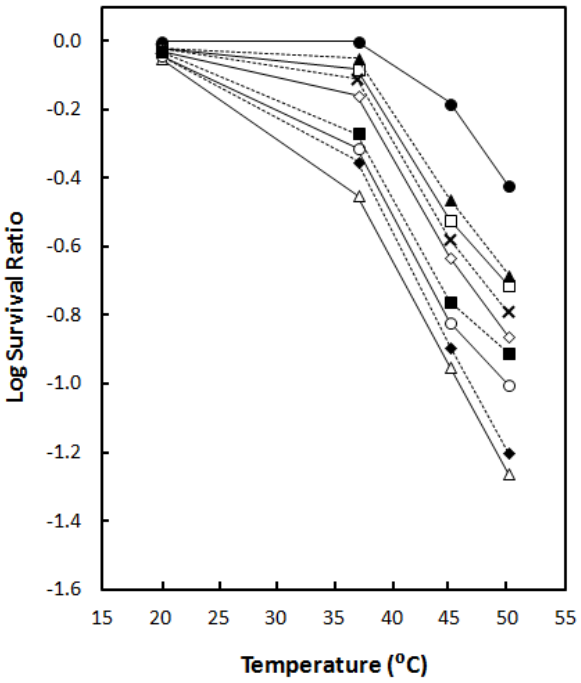

Figure 4.6 Effect of thermal stress on the bactericidal activity of CGA and related compounds against *E. coli* IFO 3301 at neutral pH, incubated at 20, 37, 45, and 50℃ for 3 h. Log survival ratio was calculated by enumerating viable cells. Data represent three independent experiments. —●—, control; —□—, chlorogenic acid; ···▲···, quinic acid; —◇—, caffeic acid; ···×···, hippuric acid; —△—, ferulic acid; ···◆···, isoferulic acid; —○—, benzoic acid; ···■···, hydroxybenzoic acid.

Table 4.2 Chemical characteristics of CGA and related compounds

Tested Compounds	FW	pK_a	LogP
Chlorogenic acid ($C_{16}H_{18}O_9$)	354.31	3.58	- 0.75
Quinic acid ($C_7H_{12}O_6$)	192.17	3.40	- 2.44
Caffeic acid ($C_9H_8O_4$)	180.16	4.47	1.15
Hippuric acid ($C_9H_9NO_3$)	179.17	3.62	0.44
Ferulic acid ($C_{10}H_{10}O_4$)	194.18	4.56	1.42
Isoferulic acid ($C_{10}H_{10}O_4$)	194.19	3.77	1.42
Benzoic acid ($C_7H_6O_2$)	122.12	4.20	1.86
Hydroxybenzoic acid ($C_7H_6O_3$)	138.12	4.08	1.49

FW, formula weight; pK_a, dissociation constant; LogP, partition coefficient.

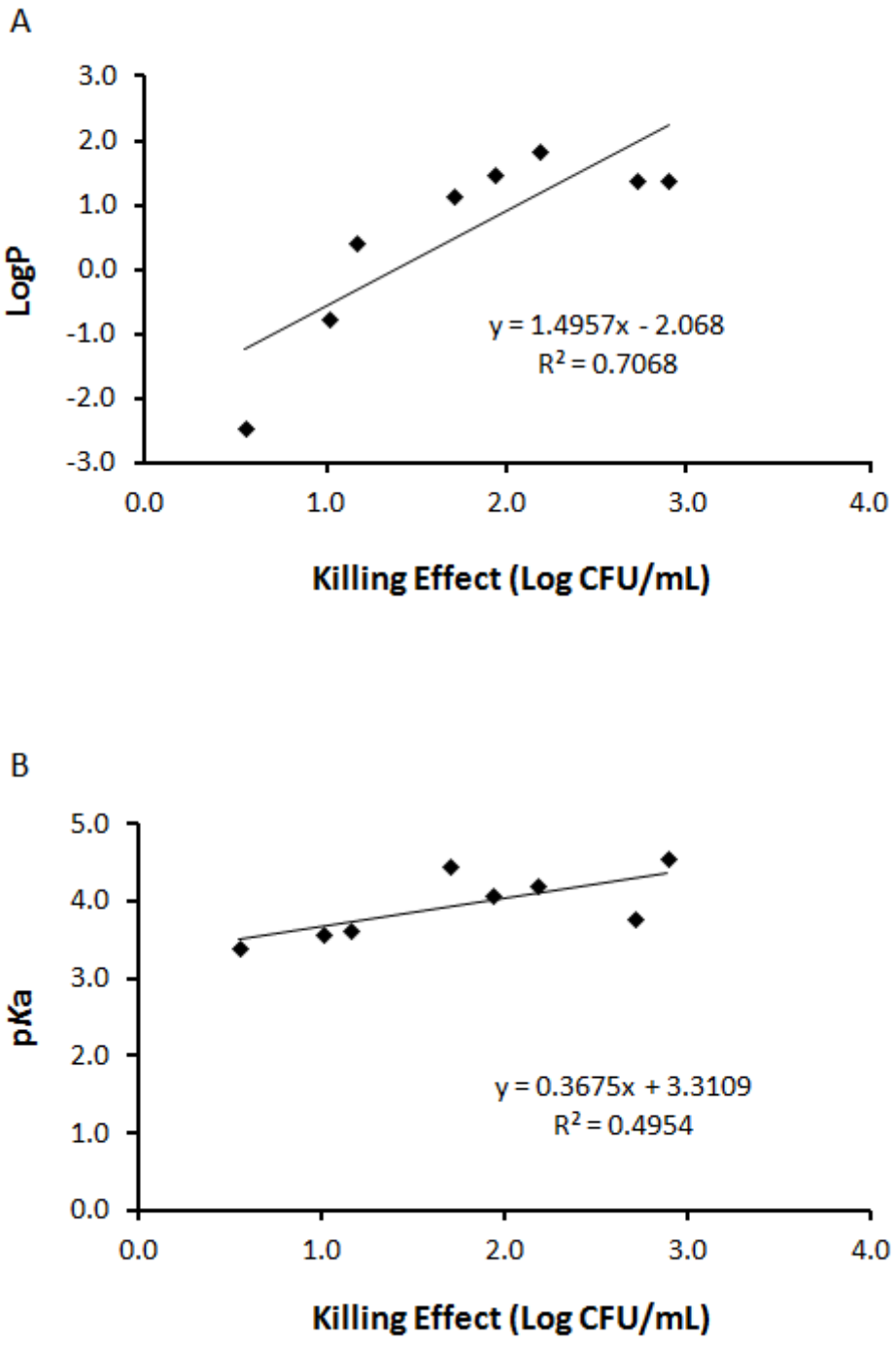

Figure 4.7 Correlation between bactericidal effects (Log CFU/mL) and LogP (A); and p*K*a (B) values.

In the present study, it was observed that CGA and related compounds possess bactericidal effects due to the action of bioactive phenolic compounds, which exerts physiological changes on the microbial cell membrane, eventually resulting in cell death. Table 4.2 shows the chemical properties of CGA and related compounds. The pK_a and LogP values of CGA and related compounds differed from each other, indicating the differences in chemical properties of each component. As shown in Fig. 7.4, the higher LogP (A) and pK_a (B) values have a tendency for improving antimicrobial activity under the neutral pH condition. pK_a values were positively (r^2=0.4954, n=8) correlated with log survival ratios after 6 h incubation. LogP values were also significantly (r^2=0.7068, n=8) correlated with the log survival ratios. Thus, it was found that the dissociation constants and partition coefficients of the tested compounds were meaningful to assess their antimicrobial actions. It was demonstrated that the combination of charge and hydrophobicity was important for antimicrobial action (73).

In contrast, it was investigated that CGA could inhibit the growth of bacteria (47). However, they did not report about the antimicrobial activities of related compounds. In the present study, a wide range of phenolic compounds were examined, not only growth inhibitory effect but also bactericidal effect. These results indicate that CGA and related compounds exhibited potent antimicrobial activities against a typical Gram-negative food-borne pathogenic bacterium and could be incorporated into various food products for which an antimicrobial additive is desired. Thus, CGA and related compounds exhibited potent antimicrobial activity, and a synergistic effect between compounds was also revealed in this study. It was proposed that these polyphenols could be useful as antimicrobial agents in the food industry during manufacturing for food safety and hygiene.

CHAPTER V

GENERAL DISCUSSION

From all of the above experimental observations, it seemed that the functional food components of agricultural wastes and by-products having antioxidant efficacy and caused severe damage to the bacteria. In this study, fruit and vegetable wastes and by-products revealed a potent antioxidant as well as antimicrobial activities against both the Gram-positive and -negative bacteria. It was observed that Gram-positive bacteria were more susceptible than that of -negative. In contrast, pure polyphenols, i.e., CGA and its related compounds have also shown strong antimicrobial activities against a typical Gram-negative bacterium *E. coli*. Most of polyphenols have greater solubility in DMSO which makes it more permeable as well as promoting its bactericidal properties. Thus, extracts from fruit and vegetable wastes and by-products contain polyphenols and these polyphenols could possibly inhibit certain enzymes involved in the bacterial fatty acid synthesis (such as FabI and FabG) which supports the notion that polyphenolic compounds have strong bacteriostatic effects and also significantly increased the permeability of outer membrane and plasma membrane that causes cells death.

In addition, the modes of action of bacterial agents depend on the type of microorganisms and are mainly related to their cell wall structure and to the outer membrane arrangement. It was indicated that the most bioactive compounds of plant extracts were more active against Gram-positive bacteria than Gram-negative bacteria (74-76). This is likely due to the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space which is not found in Gram-positive bacteria (77). The resistance of Gram-negative bacteria toward antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide (LPS) molecules, presenting a barrier to the penetration of numerous antibiotic molecules, and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (3,78). Gram-positive bacteria do not have such an outer membrane and cell wall structure.

Consequently, antibacterial substances can penetrate the bacterial cells and easily destroy the bacterial cell wall and cytoplasmic membrane and result in a leakage of the cytoplasm and its coagulation (79). Eventually, these disruptions may cause the loss of cell integrity and death. Recently, microbiologists have given attention in the

topic of antimicrobial plant extracts (80). Antimicrobial resistance is one of the prominent challenges facing global public health. Antibiotic resistance may occur via three mechanisms: prevention of interaction of the drug with target; efflux of the antibiotic from the cell; and direct destruction or modification of the compound. The increasing prevalence of multidrug resistance in pathogenic microorganisms, as well as, undesirable side effects of certain antibiotics have triggered immense interest in the search for new antimicrobial drugs of plant origin (81). Fruit and vegetable wastes and by-products are the potential sources of natural bioactive compounds. The exploitation of these abundant and low-cost renewable resources could be anticipated for the pharmaceutical as well as food industries.

Concluding Remarks

Crude extracts of agricultural wastes and by-products having functional food properties that could be beneficial for human health. Natural bioactive components of wastes and by-products possess strong antimicrobial properties. Functional food components of fruit and vegetable wastes and by-products could be useful as phytochemicals, and food preserving agents, which revealed antioxidant as well as antimicrobial potency. In contrast, CGA and related compounds exhibited potent antimicrobial activities with the synergistic effects and these polyphenols could be anticipated for the food industries during packaging and/or storage.

Suggestions for Future Research

Additional studies are needed for characterization of active compounds found in agricultural wastes and by-products and biological properties of these active extracts as a functional food components as well as application of these bioactive components as an antimicrobial agents are ongoing demand. In addition, more investigation will be needed to induce these natural bioactive components in nutraceutical formulations.

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